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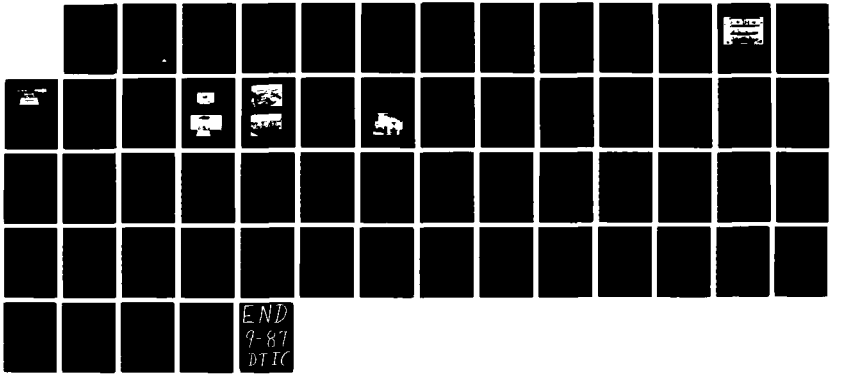

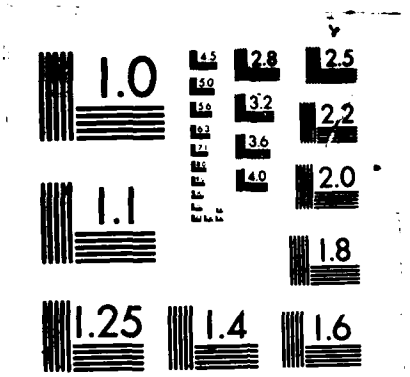

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DEFINITION OF PROCEDURE TO STUDY BIOEFFECTS OF RADIOFREQUENCY RADIATION ON CELL GROWTH AND DIFFERENTIATION

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May 1987

Final Report for Period March 1986 - October 1986

Prepared for
USAF SCHOOL OF AEROSPACE MEDICINE
Human Systems Division (AFSC)
Brooks Air Force Base, TX 78235-5301

NOTICES

This final report was submitted by Georgia Tech Research Institute, Georgia Institute of Technology, Atlanta, Georgia, under contract F33615-83-D-0601, Task No. 27, job order 7757-01-1J, with the USAF School of Aerospace Medicine, Human Systems Division, AFSC, Brooks Air Force Base, Texas. Dr. Jerome H. Krupp (USAFSAM/RZP) was the Laboratory Project Scientist-in-Charge.

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The animals involved in this study were procured, maintained, and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources-National Research Council.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

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This research program was conducted for the purpose of evaluating the feasibility of using an existing multi-animal radiation facility to expose a large population of experimental animals to electromagnetic radiation in the radiofrequency portion of the spectrum. The major conclusion of this program was that the multi-animal facility at the Georgia Tech Research Institute would provide a suitable exposure environment for investigating chronic RFR effects on the growth and differentiation of rapidly growing mammalian cells in vivo. This conclusion was reached in collaboration with experts in experimental and diagnostic pathology from Pathology Associates, Inc., Ijamsville, Maryland. The Appendix to this report presents a proposed protocol for a follow-on research program to investigate RFR bioeffects on cell growth and differentiation. This protocol identifies an animal model, animal population, exposure environment, exposure duration, and tissue assessment scheme appropriate for the proposed follow-on investigation.

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Conclusions resulting from this research were possible because of contributions from numerous persons. The authors especially appreciate the biomedical inputs on pathology from Drs. Gary Knudsen and Robert Kovatch of Pathology Associates, Inc. Excellent guidance on the cage and watering system designs, and on overall animal care and welfare, was received from the Georgia Tech Animal Welfare Committee, and from Dr. Noel Lehner, D.V.M., in particular. Finally, biometry inputs from Dr. Jerome Krupp were essential in selecting the animal subspecies and population. The important contributions of these persons to the success of this research program are gratefully acknowledged.

DEFINITION OF PROCEDURE TO STUDY BIOEFFECTS OF RADIOFREQUENCY RADIATION ON CELL GROWTH AND DIFFERENTIATION

I. INTRODUCTION

In 1926, V. I. Vernadskii observed that "We are surrounded and penetrated, at all times and in all places, by eternally changing, combining and opposing radiations of different wavelengths--from 10 millionths of a millimeter to several kilometers." Since that time, the scientific understanding of these "combining and opposing radiations" has drastically expanded, and it is now recognized that periodic electromagnetic force fields--radiations--permeate the known physical and biological environment. These force fields occur at frequencies distributed throughout the electromagnetic spectrum, and have both natural and man-made sources.

As scientific understandings of these omni-present electromagnetic radiations have increased, a concomitant interest in their interactions with biological systems has developed. This interest has derived from the fact that energetic interactions with biological systems clearly exist, and these interactions have undoubtedly played some role in the evolution of living organisms and their vital processes. Research activities aimed at understanding the interaction of electromagnetic radiations and biological systems were conducted as early as the 18th century, but it has only been in the past few decades that this research has involved rigorous interdisciplinary approaches. As a result, current research involves not only continued investigations of existing and new research approaches, but also replications of previously conducted investigations.

This research program was conducted for the purpose of evaluating the feasibility of using a multi-animal radiation facility to expose a large population of experimental animals to electromagnetic radiation in the radiofrequency portion of the spectrum. The radiofrequency radiation (RFR) of interest was an environment with sufficient power density to provide specific absorption rates (SARs) in the range of 0.1 to 0.4 W/kg for periods of time that reasonably approximate chronic exposure. Research using this environment will provide needed insights into the question of whether long-term exposure to low-level RFR influences the growth and/or differentiation of rapidly growing mammalian cells in vivo. Published reports have inferred that such RFR exposure may either act as a promoter of tumor growth or cause cells to differentiate into a more invasive form.

In conducting this research, the following major subtasks were undertaken:

1. Engineering and dosimetry efforts were undertaken to: (a) determine that accurate dose levels could be delivered, (b) assure that the exposure system could be adequately operated and monitored, (c) develop a suitable cage design, and (d) develop a reliable water delivery system that could be adapted to the exposure waveguides.

2. An animal model appropriate for a long-term study of RFR effects on cell growth and differentiation was identified. The animal model included the number, strain, biological endpoints, and endpoint evaluation parameters.

3. The need for, and a list of, Standard Operating Procedures (SOPs) was developed. Also, the key components of a Quality Assurance program were identified for a study involving a large animal population with multiple biological endpoints to be monitored in each animal.

4. An interdisciplinary team of personnel suitable for conducting the long-term study was identified, and a plan for support and logistics was developed.

Results from these subtasks are presented in the following four sections of this report. These results clearly indicated the feasibility of using an existing multi-animal radiation facility to chronically expose a large population of experimental animals to low-level RFR to investigate radiation influences on the growth and differentiation of rapidly growing mammalian cells in vivo. Therefore, a long-term study of low-level bioeffects is recommended, and a detailed protocol for this proposed study is included as an Appendix to this report.

II. ENGINEERING AND DOSIMETRY EFFORT

Radiation Facility Investigation

The initial engineering studies involved evaluating the feasibility of using an existing multi-animal radiation facility to expose a large population of experimental animals to determine RFR effects on cell growth and differentiation in vivo. This facility consisted of an 8-room complex in the basement of the Baker Building on the main Georgia Tech campus. The eight contiguous rooms were identified as follows (Figure 1):

• Room A	Radiation Room	7.62 m x 7.62 m	(25 ft x 25 ft)
• Room B	Control Room	7.62 m x 7.62 m	(25 ft x 25 ft)
• Room C	Caretaker's Office	2.44 m x 3 m	(8 ft x 10 ft)
• Room D	Cage Washer Room	2.44 m x 3.6 m	(8 ft x 12 ft)
• Room E	Weigh Room	2.44 m x 3 m	(8 ft x 10 ft)
• Room F	Assay Room	2.44 m x 2.44 m	(8 ft x 8 ft)
• Room G	Buffer Room	1.8 m x 9.14 m	(6 ft x 30 ft)
• Room H	Transmitter Room	1.8 m x 7.62 m	(6 ft x 25 ft)

The Radiation and Control Rooms were in essence shielded anechoic test chambers in that their walls were lined with microwave absorbing material with a reflectivity greater than -25 dB at 500 MHz. Both the walls to which the absorbing material was attached and the ceilings were shielded with metal foil. The floors did not require either shielding or absorbing material since they were on the ground level of the Baker Building. The Caretaker's Office provided desk space for record keeping and file updating. Also, storage space for the various supplies and materials used in maintaining and cleaning the Facility was provided in this office. The Cage Washer Room housed a Southern Cross Model Cage Washer with stainless steel entrance and exit tables. The cage washer offered timed wash and rinse cycles and a cycle during which disinfectants could be added to rinse water. Temperature of the wash water could be adjusted from room temperature up to 180 ^{o}C. Metal racks used with the cage washer were designed to accommodate large cages of the type used to house rats, but the washer would easily accept new racks designed to accept cages for either larger or smaller animals. The Weigh Room had a Sartorius Model 1203 MP Electronic Balance with a keypad that was electronically interfaced with a Cromemco Model Z-2H Computer located in the Buffer Room. Therefore, animals could be rapidly weighed a preprogrammed number of times, the average weight could be calculated, and the average weight plus the animal number could be electronically transferred to the computer. Once in the computer, software was available for conducting a variety of statistical analyses. The Assay Room was located in an area that was extremely quiet; therefore, animal examinations could be conducted without introducing artifacts due to external noise and/or activity. Isolation

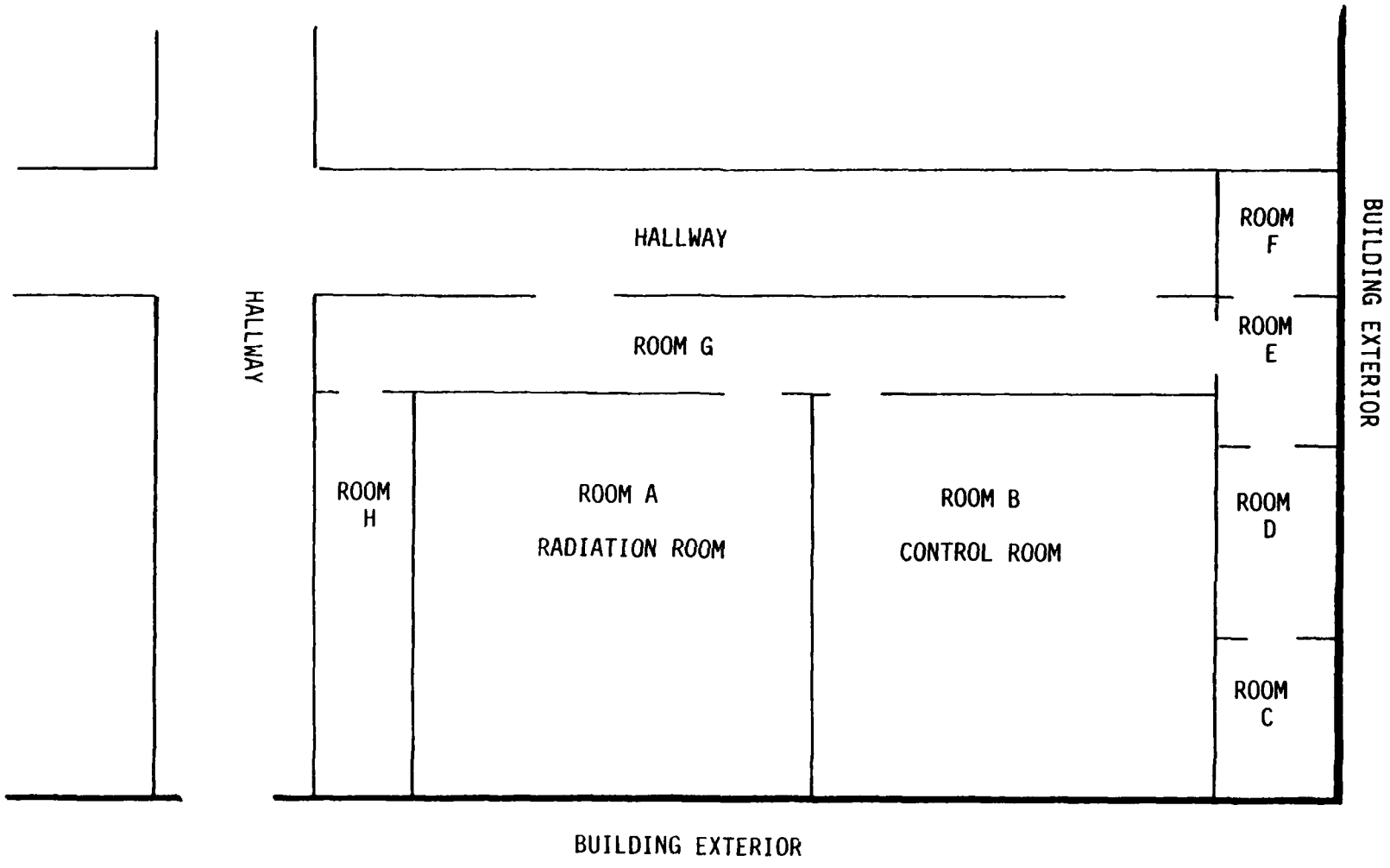


Figure 1. Floor plan for existing Radiofrequency Radiation Facility in Room 19, Baker Building, on Georgia Tech Main Campus.

of the facility from activities in the remainder of the Baker Building was provided by the Buffer Room. The Transmitter Room housed a high-power 435-MHz transmitter capable of operating in either the pulsed- or continuous-wave (CW) mode. Outputs from this transmitter were routed up through the ceiling and then to the feed antennas in the Radiation Room. Special cooling in the Transmitter Room was provided by an exhaust to outside air, thus prohibiting heat accumulation in the room due to transmitter operation.

Air conditioning and heating provisions for the entire 8-room complex were separate from the remainder of the Baker Building; therefore, temperature conditions in the facility could be maintained independent of other activities in the building. Also, the Radiation and Control Rooms had timer-controlled lighting systems; therefore, the day/night cycle could be adjusted as desired for the study purposes or as convenient for the facility's operation.

The exposure devices in the Radiation and Control Rooms were open-ended waveguides constructed of four sets of circular, parallel plates stacked one set above the other as shown in Figure 2. The plate diameters were 3.66 m (12 ft) and the separation distance between plates was 45.7 cm (18 in.). These four exposure waveguides were fed by slotted-cylinder antennas located at their centers. The 45.7-cm (18 in.) separation distance assured that only the lowest order TE-mode wave propagated outward in concentric circles about the slotted-cylinder feed antennas. Use of slotted-cylinder feed antennas assured a horizontally polarized exposure field with an amplitude in the azimuth plane that was nearly constant around the waveguide circumference. The horizontal polarization meant that the electric field vector would be parallel to the long dimension of most any experimental animal selected for later RFR bioeffects studies. This orientation assured maximum coupling of the exposure field to the animal. The electric field vector was necessarily zero at the surface of the metal plates, thereby preventing mutual coupling between adjoining sets of parallel plates. Extensive radiation pattern measurements had been made to define the radiation characteristics of this facility. The resulting data confirmed TE-01 mode propagation in which the vertical component of the electric field vector was typically 17 dB below the horizontal component at 435 MHz.

The four slotted-cylinder antennas used to feed the circular, parallel plate waveguides were connected to the high-power transmitter through impedance-matching devices--baluns--to maximize the transfer of 435-MHz transmitter power to the antennas. These baluns were specially designed to both withstand the heat generated by the transmitter signal and to provide the necessary impedance transformations. During previous operation of the exposure facility, frequent readjustments of the transmitter output were necessary, indicating that impedance matching between the transmitter and antennas was changing as a function of time. During this program, these baluns were removed from the slotted-cylinder waveguides and visually examined. This examination revealed considerable corrosion of the baluns, probably due to heat. A balun design capable of withstanding more heat was developed and should be used during later studies that require exposure power densities in the range of 1.0 mW/cm^2.

Since the diameter of the plates forming the exposure waveguides in the Radiation and Control Rooms was 3.66 m (12 ft), the circumference of each of the 8 plates (4 in the Radiation Room and 4 in the Control Room) was 1148 cm (452 in.). Therefore, if the width of animal housing units plus the intercage separation distance were 22.9 cm (9 in.) or less (as would be appropriate for

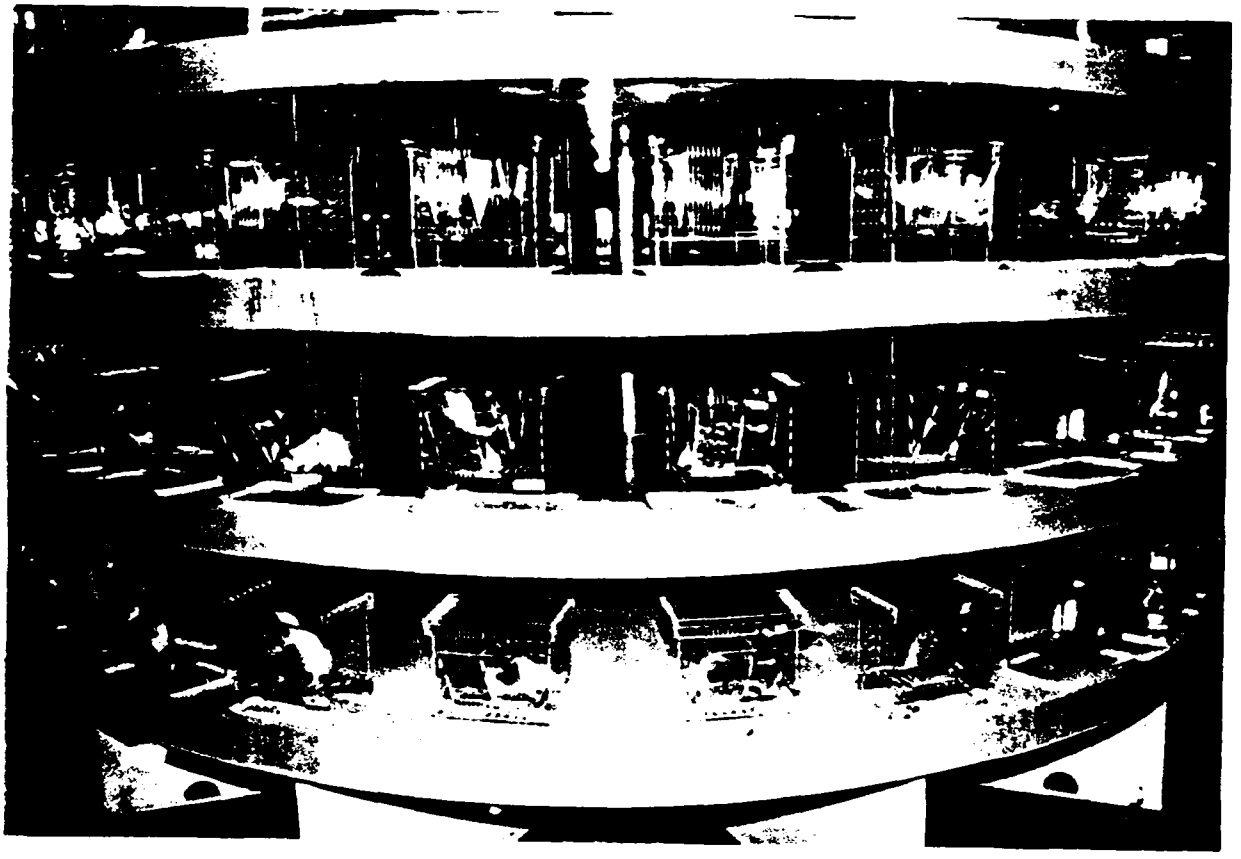


Figure 2. Rats positioned on circular, parallel-plate waveguides.

mice), 50 cages could be positioned on each waveguide. This arrangement would mean that 200 exposure and 200 sham-exposure animals could be accommodated in a long-term study of low-level RFR effects on cell growth and differentiation in vivo.

Cage Development

During this program, cage designs suitable for use in long-term studies of RFR effects were developed. As the designs evolved, they were frequently reviewed by the chairman of, and veterinarian on, the Georgia Tech Animal Welfare Committee. Additionally, the designs were periodically reviewed by Drs. Robert Kovatch and James Stevens of Pathology Associates, Inc. (PAI), and on 24 July 1986, Dr. Jerome Krupp of the USAF School of Aerospace Medicine reviewed designs for both the cages and the watering system.

Throughout this development effort, it was assumed that the animal model selected for later RFR effects studies would be the mouse; however, the resulting designs could be adapted to other models by simply changing appropriate physical dimensions.

Animal housing is an extremely important, and sometimes overlooked, facet of long-term RFR studies. The importance of housing stems from the fact that it, as much as any other factor, defines the animal's physical environment for the duration of the study; therefore, housing directly influences the health, well-being, and behavior of the animal, and if inadequately considered, can introduce artifacts in the study results.

During this effort, factors considered important in adequate housing for mice were [1]:

- space that permitted freedom of movement, normal postural adjustments, and an appropriate resting area,
- an escape-proof enclosure that safely confines the animal,
- adequate ventilation,
- biological needs of the animal such as maintenance of body temperature, urination, and defecation,
- cleanliness, and
- protection from known hazards.

With these factors in mind, the initial cage design shown in Figure 3 was developed. Major features of this design were Plexiglas material that provided RFR transparency at 435 MHz, a 7.62 cm × 12.7 cm (3 in. x 5 in.) floor area, a cage height of 12.5 cm (5 in.), a food hopper that extended into the cage from the top, a floor and ceiling constructed of 0.317 cm (0.125 in.) glass dowels oriented perpendicular to the cage long dimension, and a watering sipper tube that entered the cage from the side. The orientation of the glass dowels was intended to induce the mice to align themselves preferentially with their long axes parallel to the exposure field.

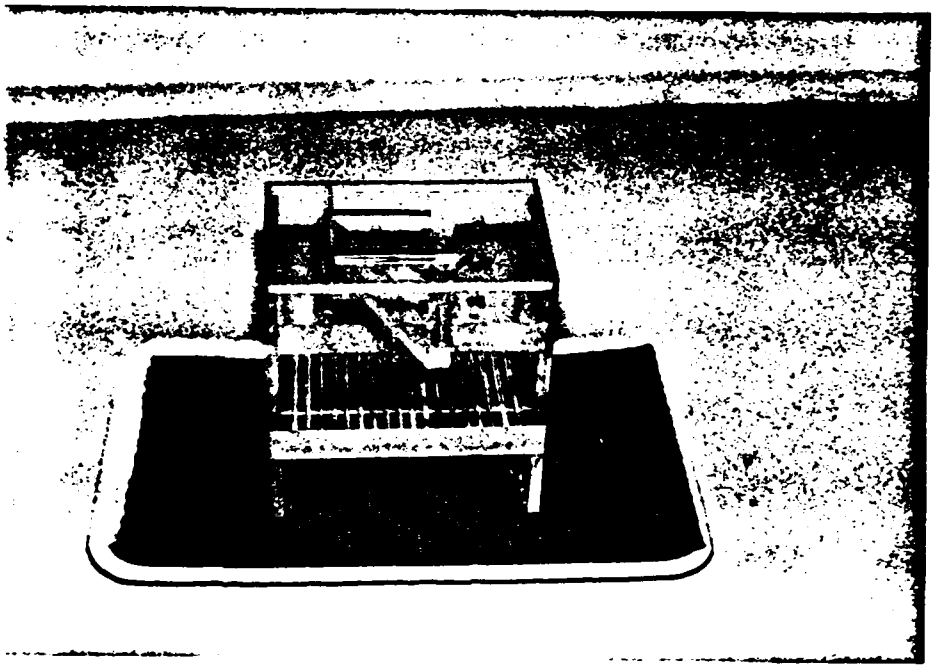


Figure 3. Initial cage design.

After reviewing this design with the Georgia Tech Animal Welfare Committee and with Drs. Kovatch and Stevens, 10 prototype cages were constructed for evaluation. The evaluation consisted of observing the health, well-being, behavior, and orientation preferences of mice housed individually in the cages for a 2-week time period. Results of this 2-week evaluation were satisfactory; however, members of the Animal Welfare Committee expressed concern with the amount of floor area since the cages were to be used in long-term studies. Consequently, the width dimension of the floor was increased from 7.62 to 12.7 cm (3 to 5 in.), thereby yielding a square floor with an area increase of approximately 67%. This width increase was small enough that 50 cages could still be positioned around the circumference of the exposure waveguides in the Radiation Facility.

In addition to concern about the floor area, members of the Animal Welfare Committee also expressed concern about the height of the cages. This concern focused on the fact that the food hopper extended 7.62 cm (3 in.) down into the cage, and was therefore only 5.08 cm (2 in.) above the cage floor. This location of the food hopper limited the cage's usable volume and could possibly induce artifacts. Therefore, the food hopper was relocated to the cage side, and the cage top was redesigned to consist of a Plexiglas frame with glass dowels in its center.

Watering System Development

The development of a watering system that would reliably serve 200 mice without perturbing the exposure field required a major design effort. Design criteria required that the system

- contain no metal parts,
- be leak proof,
- deliver water on a gravity-feed basis,
- be easily disconnected,
- be capable of complete sterilization,
- be capable of being mounted on the exposure waveguide,
- not perturb the exposure field,
- be able to accommodate low pH water, and
- be opaque.

As an initial conception, the watering system was envisioned as a set of 4 reservoirs positioned 90 degrees apart and located on top of the stack of parallel plate waveguides. A feed line from each reservoir would branch into multiple lines to serve individual cages. Each reservoir would provide sufficient water to its branches to last approximately 5 days.

A single branch of this system, capable of servicing up to 64 cages, was constructed on the Radiation Room waveguide structure to evaluate its performance. Flow in this system was gravity fed, and Nalgene stopcocks were provided to achieve a controlled flow into glass sipper tubes mounted in the cage sides.

The evaluation of this watering system indicated it to be unacceptable because of the inability to provide the correct flow to individual sipper tubes over a long period. Too frequently, the incorrect flow led to the delivery of either too much or too little water to the cage.

Efforts to redesign the watering system first focused on a replacement for the glass sipper tubes. This replacement meant finding a glass or plastic analog for the commonly used "LIXIT" valves used in most automatic watering systems. A thorough survey of vendor literature and contacts with companies specializing in watering systems indicated that suitable analogs to the "LIXIT" valves were not readily available. However, an all-plastic watering valve used extensively in poultry farming was identified and appeared to be adaptable for this application. These valves, manufactured by Hart Systems of Glendale, California, and known as Hartcups, were gravity fed (operating between 1400 and 14000 kg/m^2; 2 and 20 $lbs/in.^2$) and were trigger, rather than float, operated.

The Model C-5X-B Hartcup appeared usable for this program, so sample valves were obtained. The cup shape permitted easy attachment to the sides of the Plexiglas cages. During evaluations, a mouse would drink by pushing the trip-lever in the cup with its nose. The trip-lever opened a polymer seal external to the cup, thus permitting water to flow into the cup. The mouse would then drink the water deposited on a curved bottom surface in the cup.

Use of the Hartcup required another change in the cage design as shown in Figure 4. Fifteen cages of this modified design were then obtained for evaluation with the final design of the watering system. This final watering system design is shown in Figure 5 and offered the following features:

- Central reservoirs, consisting of two 15.2-L (4 gallon) Nalgene "Lowboy" carboys, were located one each on top of the two circular, parallel plate waveguide assemblies.

- The two carboys fed 0.953-cm (0.375 in.) diameter Norton Norprene thermoplastic elastomer tubing that distributed the water to the desired cage locations. Norprene is a high quality, flexible, black tubing possessing excellent chemical resistance.

- The Norprene tubing circled the underside perimeter of each plate (except the bottom one) in the two exposure waveguide assemblies. Nalgene "T" fittings connected "drop" tubing that channelled water from the perimeter ring down to pairs of cages. Each ring connected with the ring directly above and below it; therefore, the system provided continuity from top to bottom plate.

- Adhesive tie mounts attached to the underside perimeter of the waveguide plates provided locations for attaching the plastic distribution and "drop" tubing.

- The perimeter tubing of one tier of waveguide could be isolated from the tubing of another tier by closing one-way needle valves positioned in the tubes interconnecting the tier levels.

- Each "T" on the perimeter branched into two lines through a "Y" connector, thus serving two cages.

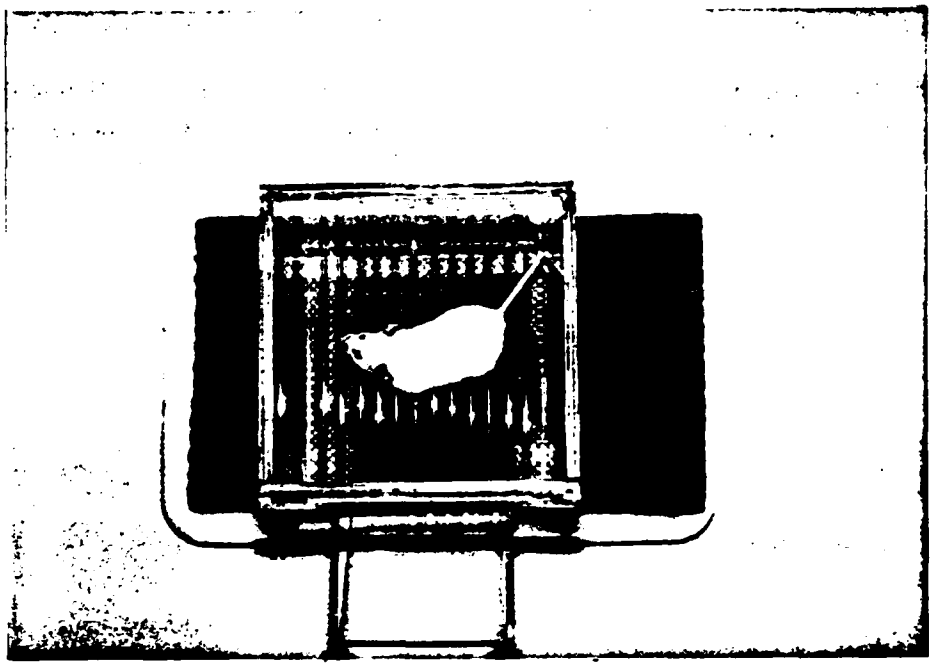
- Small plastic pinch clamps directly ahead of the cage Hartcups disconnected water flow to each cage, thereby preventing leakage when cages were removed.

- Cable ties reinforced all tubing joints, thus minimizing leaks.

This program did not provide an opportunity to evaluate the effectiveness of ultraviolet lamps in sterilizing the watering system; however, consultations with persons working with watering systems indicated the possible need for such lamps. These lamps are said to be capable of a 99.9 % "kill" ratio, and would reduce the need to acidify the system water. This method would benefit the animals, staff technicians, and useful lifetime of the watering system.

Dosimetry Studies

Over the past decade, there has been an overwhelming realization that absorbed dose rather than incident power density or field intensity is the independent variable of interest in bioeffects studies. Absorbed dose



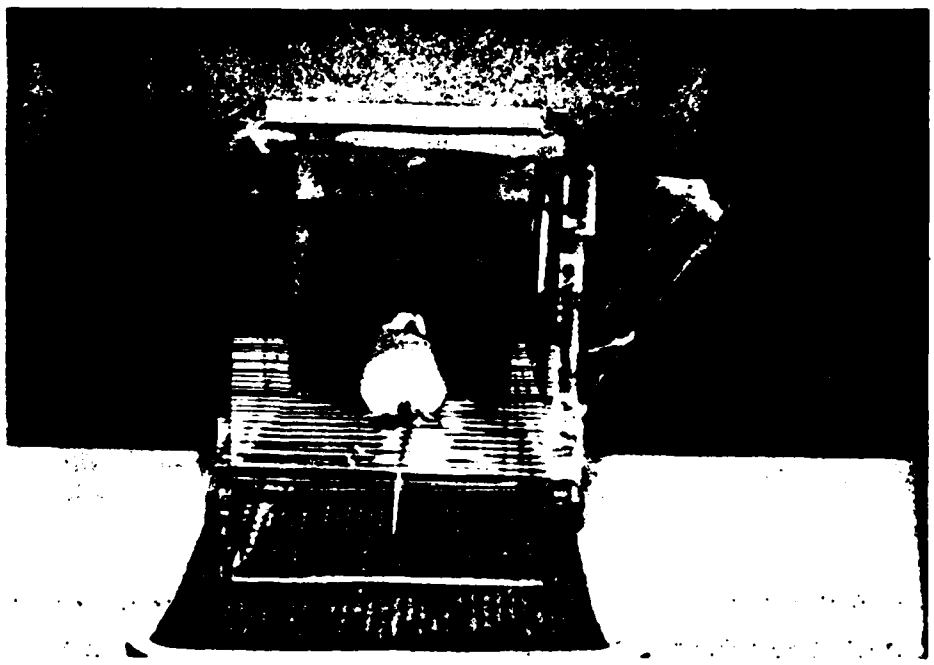
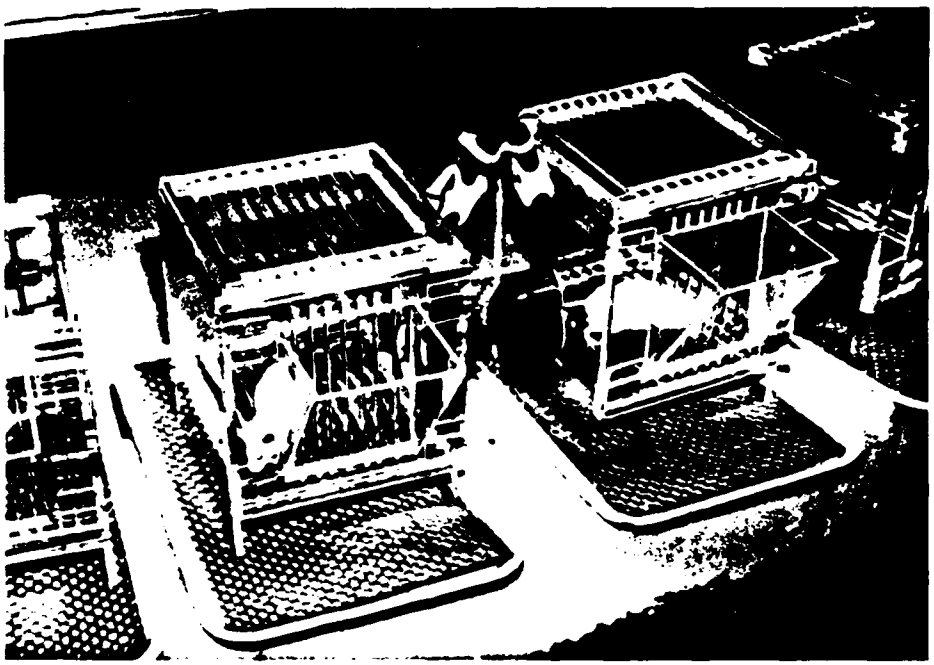


Figure 4. Final cage design.



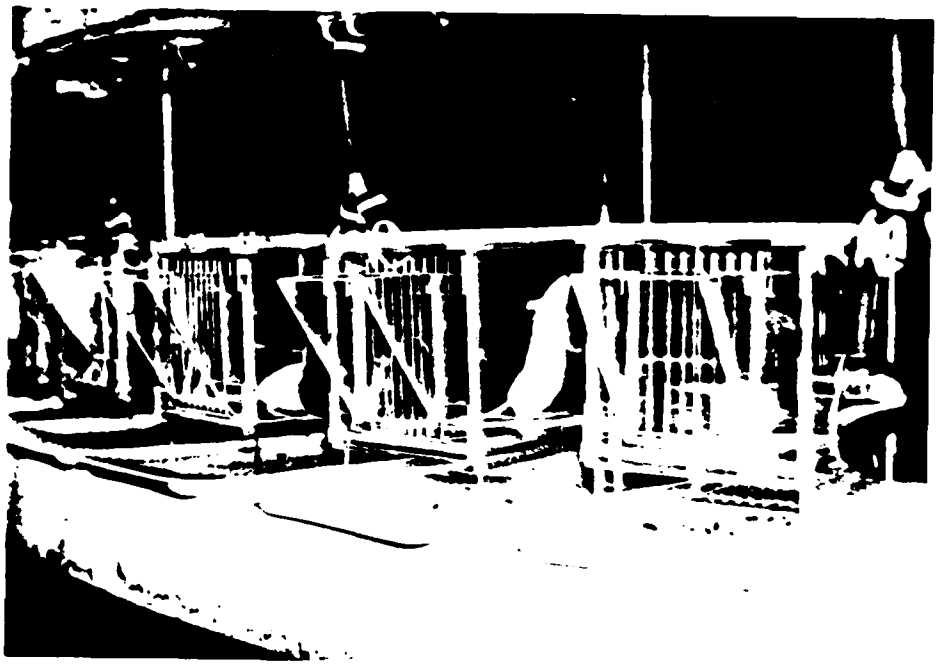


Figure 5. Watering system design.

represents a factor which can be accurately scaled to account for variations in either RF frequency, incident power density/field intensity, or test subjects. Without adequate dosimetric information, experiments can not be adequately replicated, nor can exposure results obtained using experimental animals be extrapolated to equivalent human models.

The circular, parallel-plate waveguides in the just described multi-animal exposure facility generated a horizontally polarized exposure field. Therefore, if an experimental animal were aligned such that its long dimension were parallel to the electric field vector (generally referred to as EHK polarization), maximum coupling between the exposure field and the animal would occur. Under this condition, if (1) the animal were then assumed to be a medium-sized mouse that was modelled as an ellipsoid, (2) the exposure field were assumed to have a frequency of 435 MHz, and (3) the incident power density were assumed to be 1.0 mW/cm^2, then the theoretical absorbed dose would be approximately 0.1 W/kg [2]. Since this absorbed dose was theoretically determined, it was necessary during this program to accurately measure the absorbed dose using actual experimental conditions.

To determine the absorbed dose, or the SAR, induced in an animal exposed to the field generated by the circular, parallel-plate facility, a series of calorimetry measurements was undertaken. These measurements were a rather straightforward application of the Law of Energy Conservation, which states that the total energy within a closed system remains constant. To determine the total energy absorbed by an animal carcass, a calorimeter was first allowed to reach steady-state conditions. The animal carcass was then exposed to the radiation field and subsequently introduced into the calorimeter. Measuring the resulting change in temperature within the calorimeter permitted the energy absorbed by the carcass to be determined. The equation that described the energy exchange of interest was

$$E = MC(T_e - T_b), \tag{1}$$

where E = energy absorbed by the carcass,
M = mass of the carcass,
C = composite specific heat of the carcass,
T_e = postexposure temperature of the carcass, and
T_b = preexposure temperature of the carcass.

As noted, T_b and T_e represent the before and after steady-state core temperature of the animal carcass. If the carcass temperature were allowed to stabilize over a several hour period of time, then T_b would equal the ambient temperature T_a. The postexposure temperature could be determined two ways, the most straightforward of which would involve inserting a temperature probe into the carcass and measuring temperature during radiation exposure. However, this approach was not suitable for this program because the length of the available nonperturbing temperature probe would not extend beyond the exposure field area.

The second method for measuring the postexposure temperature involved using a calorimeter. The equation

$$(M_s C_s + M_{cal}\, C_{cal})\,(T_e - T_f) = (Z_d + M_{bar}\, C_{bar} + M_{H_2O}\, C_{H_2O})\,(T_f - T_i) \tag{2}$$

where M_s = mass of the carcass (g),

C_s = composite carcass specific heat (0.824 cal/g·°C for a mouse),
M_{cal} = mass of the calorimeter (98.3 g),
C_{cal} = specific heat of the calorimeter,
M_{bar} = mass of the magnetic stirring bar (11.2 g),
C_{bar} = specific heat of the magnetic stirring bar (0.16 cal/°C·g),
M_{H2O} = mass of water in the calorimeter (125 g),
C_{H2O} = specific heat of the water (defined as 1.0 cal/°C·g),
Z_d = calorimeter heat capacity,
T_e = postradiation temperature of the carcass,
T_f = final water temperature in calorimeter,
T_i = initial water temperature in calorimeter

could then be used to solve for the postradiation core temperature T_e.

To use this equation, a modified Dewar calorimeter was constructed [3] from available laboratory materials. A well for the carcass container was cut into a piece of 10.16 cm (4 in.) thick Styrofoam material. The well's dimensions provided a tight fit for a 250-mL Pyrex beaker. A second piece of 10.16 cm (4 in.) thick Styrofoam was cut to provide a top for the calorimeter. Both Styrofoam pieces were then cut to fit in a 15.24 cm (6 in.) diameter thin-metal sleeve. The Styrofoam top was drilled to accept a Vitek Model 101 Electrothermia Monitor probe to measure the temperature of the beaker's contents. Two hollow glass dowels with long threaded screws running inside provided a method for clamping the top and bottom Styrofoam pieces together. The final calorimeter is shown in Figure 6.

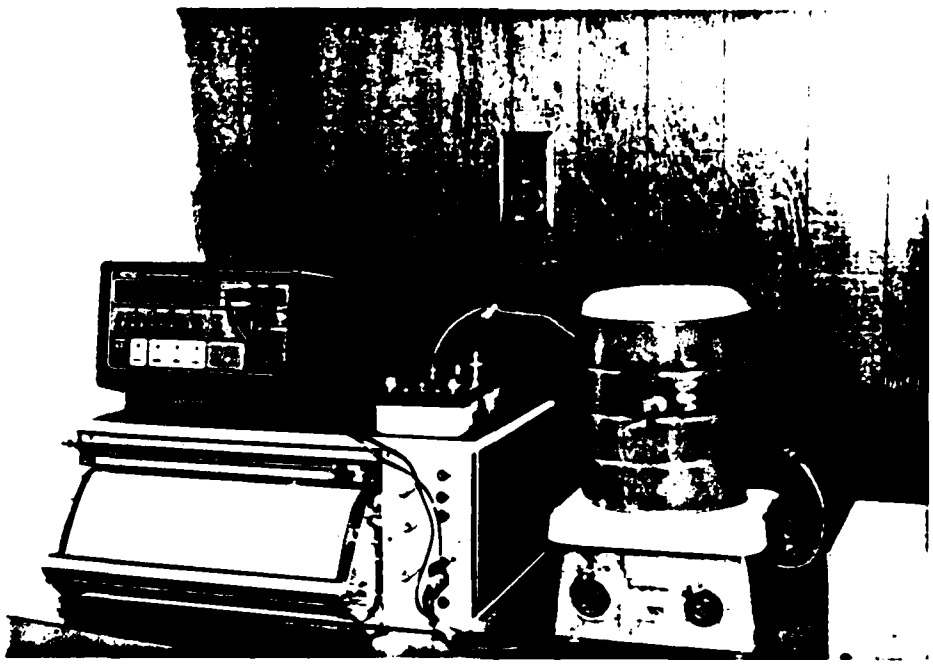


Figure 6. Calorimetry configuration for dosimetry determinations.

The calorimeter's specific heat had to be determined before it could be used for SAR measurements. The equation used to calculate this specific heat was

$$E = (M_{cal}C_{cal} + M_{H_2O} + M_{bar}C_{bar})\,(T_f - T_i), \qquad (3)$$

where E is the total energy dissipated into the calorimeter, and the other terms are as previously defined.

To obtain a controlled energy dissipation in the calorimeter, a 0.25-W, 1.49 kilohm resistor was submerged in the distilled water used to fill the 250-ml beaker in the calorimeter. The point where the resistor leads and wire touched was treated five times with a waterproofing sealant to prevent electrolysis of the water. The magnetic stirring rod at the bottom of the beaker maintained a uniform temperature within the calorimeter. Temperature was monitored 30 minutes before energizing the resistor (to assure that the calorimeter has attained a steady-state initial temperature), 30 minutes while the resistor was energized, and then 30 minutes after the resistor was de-energized (to assure the calorimeter reachieved a steady-state temperature). The Vitek Monitor output was recorded on a Hewlett-Packard Model 7132A Strip Chart Recorder providing a full-scale reading (250 divisions) covering a range of 1 ^{o}C. With a voltmeter and power supply, a controlled dissipation of 0.1987 W was obtained from the resistor. Three measurement series were conducted to determine the temperature difference ($T_f - T_i$), now labeled ΔT, resulting from the submerged resistor. The measurement series produced ΔTs of 0.3320, 0.3520, and 0.3220 ^{o}C. Using the mean of these ΔTs as the quantity ($T_f - T_i$) in equation (3) yielded a C_{cal} of 1.0493 cal/^{o}C.g.

With the specific heat of the calorimeter determined, it was then possible to perform calorimetry measurements. Since the theoretical SAR values previously obtained from dosimetry curves were given for a 1.0 mW/cm^2 exposure field, it was desirable to conduct the calorimetry measurements in a field of the same power density. This procedure required calibrating the field in the exposure facility. A calibrated dipole antenna (Scientific Atlanta Model 15-350) tuned to 435 MHz was placed midway between one set of circular, parallel plates. The position was the same location where cages housing experimental animals would subsequently be placed. The antenna was oriented tangent to the circumference of the plates and then connected through a coaxial cable to a power meter located outside the Radiation Room. Losses in this coaxial cable were determined and used in the power calculations. Appropriate calculations were made to determine the power level that would be indicated by the power meter (31.11 dB) when the exposure field was 1.0 mW/cm^2.

The procedure for each calorimetry measurement was as follows. On the day before the measurement, a test mouse was chosen and euthanatized using chloroform asphyxiation. The carcass was weighed and then placed in a prototype Plexiglas cage on the waveguide perimeter overnight to assure that thermal equilibrium with the ambient temperature of the room was achieved. Simultaneously, the calorimeter was filled with 125 g of water that was allowed to equilibrate overnight.

The following morning, the exposure facility was energized and the carcass was exposed to a power density of approximately 1.0 mW/cm^2 for 30 minutes. Just before termination of the exposure, the calorimeter was prepared and calibrated for receipt of the carcass. The water temperature within the calorimeter was measured and checked to be approximately 1 to 2 ^{o}C below ambient temperature [4]. If this was not the case, the water was cooled by immersing a bag containing ice; therefore, water above the 125-mL mark was not added to the beaker.

The field was then deenergized, and the mouse carcass was immediately transferred into the calorimeter. Temperature within the calorimeter was then monitored until the system reached steady-state conditions. The temperature difference ΔT was then taken from the strip charts and used in equation (2) to calculate the mouse core temperature immediately after exposure to RFR. This temperature in turn was used in equation (1) to calculate the energy absorbed by the carcass.

This procedure was repeated numerous times over a 2-week period of time. The mean value of the resulting SAR levels was 0.32 W/kg. The absorbed dose was achieved without the transmitter operating at its maximum output level.

III. SELECTION OF ANIMAL MODEL AND EXPOSURE DURATION

The selection of an animal model and an exposure duration appropriate for studying low-level RFR bioeffects on rapidly growing mammalian cells in vivo was one of the most important efforts of this program; therefore, assistance from scientists with recognized expertise in this area was obtained by means of a subcontract with Pathology Associates, Inc. of Ijamsville, Maryland.

Pathology Associates, Inc.

Pathology Associates, Inc. (PAI) is an independently owned small business specializing in biomedical research support services. The company was founded in March 1981 and has its corporate offices in Ijamsville, Maryland. Full-service commercial histopathology laboratories are operated in Maryland, Ohio, North Carolina, and Massachusetts, while specialized service laboratories are operated in Arkansas and Illinois. The company has gained recognition as a responsive scientific organization providing services in support of research programs sponsored by both federal agencies and private-sector clients. The company has also a proven record in the management and operation of large-scale, sophisticated biomedical support activities and has corporate experience in performing high-volume, production-oriented services as well as in providing collaborative consultation in several biomedical research areas.

Currently, PAI is responsible for more than 100 separate projects involving various aspects of rodent in vivo toxicology, reproductive toxicology, and carcinogenesis bioassay. Generally, their services include conducting and supervising necropsies, all aspects of routine histopathology, and clinical pathology. Additionally, they provide routine histopathology and a wide variety of special experimental pathology techniques in support of research and development programs. These services include all aspects of electron microscopy (transmission and scanning), histo- and immunohisto-chemistry, plastic sectioning, autoradiography, and morphometry. In all cases, the routine and specialized services are conducted in accordance with Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) Good Laboratory Practice regulations.

The senior staff at PAI is predominantly oriented toward pathology with 20 full-time pathologists. However, the staff also includes members with expertise in teratology, clinical pathology, laboratory animal medicine and husbandry, and Good Laboratory Practice/Quality Assurance requirements.

Although the PAI professional staff is predominantly oriented toward pathology, they are also extensively involved in the performance of life sciences services that integrate histopathology with clinical pathology, facilities management, laboratory animal medicine/husbandry, rodent carcinogenesis and toxicology, teratology, electron microscopy, and quality assurance.

When PAI was contacted regarding contributions to this program, Robert M. Kovatch, D.V.M., was identified as the principal contact. Dr. Kovatch is a Diplomate, American College of Veterinary Pathologists (ACVP), with more than 20 years experience in various aspects of experimental and diagnostic pathology.

He is the Project Manager for pathology support for the National Cancer Institute (NCI) Federal Cancer Research Facility (FCRF) research program at Fort Detrick, Maryland. He also serves as Head, Quality Assurance (QA) Pathologist as part of PAI's internal quality assurance program. Dr. Kovatch has received international acclaim as a rodent pathologist and has evaluated the effects of more than 100 chemicals in rodent bioassay test systems. He served as either Department Chief or Senior Pathologist at five different U. S. Army biological research institutes including Director, Division of Pathology at the Walter Reed Army Institute of Research. He has reviewed (quality assured) over 40 subchronic studies, chaired two Pathology Working Groups (PWG), and participated as a PWG member on 24 bioassay studies. Dr. Kovatch has evaluated an extensive number of acute, subchronic, and chronic rodent studies and has numerous publications in the area of rodent carcinogenesis.

Selection of Animal Model and Exposure Duration

Selection of the specific animal model for the proposed bioeffects study was influenced by (1) results of a previous USAF-sponsored bioeffects study [5], (2) the need to validate the findings of Szmigielski et al. [6], and (3) the desire to strengthen the statistical significance of the study results by using the largest possible number of animals. Use of a large animal population had to take into account the dimensions and capabilities of the existing exposure facility.

The previous USAF-sponsored bioeffects study used male Specific-Pathogen-Free Sprague Dawley rats. Although this study indicated an apparent increased incidence of malignant neoplasms in exposed versus sham-exposed rats, no single tissue or organ system developed a significant increase in benign or malignant neoplasms. The study by Szmigielski et al. used female C3H mice as the test animal and reported a slight increase in mammary tumors plus an accelerated development of spontaneous- and benzopyrene-induced skin cancer. In this study, the RFR level was greater than that used in the USAF-sponsored study.

The study by Szmigielski et al. raised the concern that long-term RFR exposure may affect the mammary tissues of female mice. Correlations between this study and the USAF-sponsored study could not be made because different animal species, animal sexes, and exposure fields were used. To determine the significance of the Szmigielski et al. results, and to gain a broader insight into RFR effects on cell growth and differentiation in general, it was logical to conduct future studies using C3H mice.

The murine mammary tumor virus (MuTv) C3H/HeJ mouse was selected as the preferred animal model for studying the bioeffects of RFR exposure on cell growth and differentiation. This selection was supported by substantial amounts of data that exist on this particular substrain of mouse. In particular, mammary tumors in the C3H mouse are among the most frequently studied spontaneously occurring neoplasms, and the incidence and time of occurrence in the C3H mouse are well documented in the literature. For example, Outzen et al. [7] recently reported the mammary tumor incidence and latency in the C3H/HeJ substrain maintained at the Jackson Laboratory. The average time required for 50 % incidence of mammary tumors in the C3H mouse was 61 weeks of age. Also, the use of a mouse as the animal model would mean that 400 animals (200 exposed, 200 sham exposed) could be used with the existing Radiation Facility. As shown

in the Figure 7 operating curve, this number of animals would permit a 15% change to be detected, if it existed, 90 % of the time.

Therefore, 400 (plus any sentinel animals desired) female C3H/HeJ mice should be obtained from a commercially acceptable vendor shortly after weaning. The mice should be held in quarantine for a 2-week period and then be introduced into the exposure schedule at approximately 5 weeks of age. The exposure schedule should be terminated after 18 months, with the animals that live to term being approximately 77 weeks of age. More than 50 % of these mice will have developed mammary tumors by this time.

In summary, 400 mammary-tumor-sensitive C3H/HeJ mice should be used for further bioeffects studies of cell growth and differentiation in vivo. This animal model permits the significance of the Szmigieski et al. study and, in particular, the RFR sensitivity of mammary tissues in female mice, to be thoroughly evaluated and/or validated. Also, selection of this animal model allows a large population of exposure and sham-exposure animals to be used, thereby assuring statistical analyses in which a 15 % change in tumor incidence can be detected, if it exists, 90 % of time.

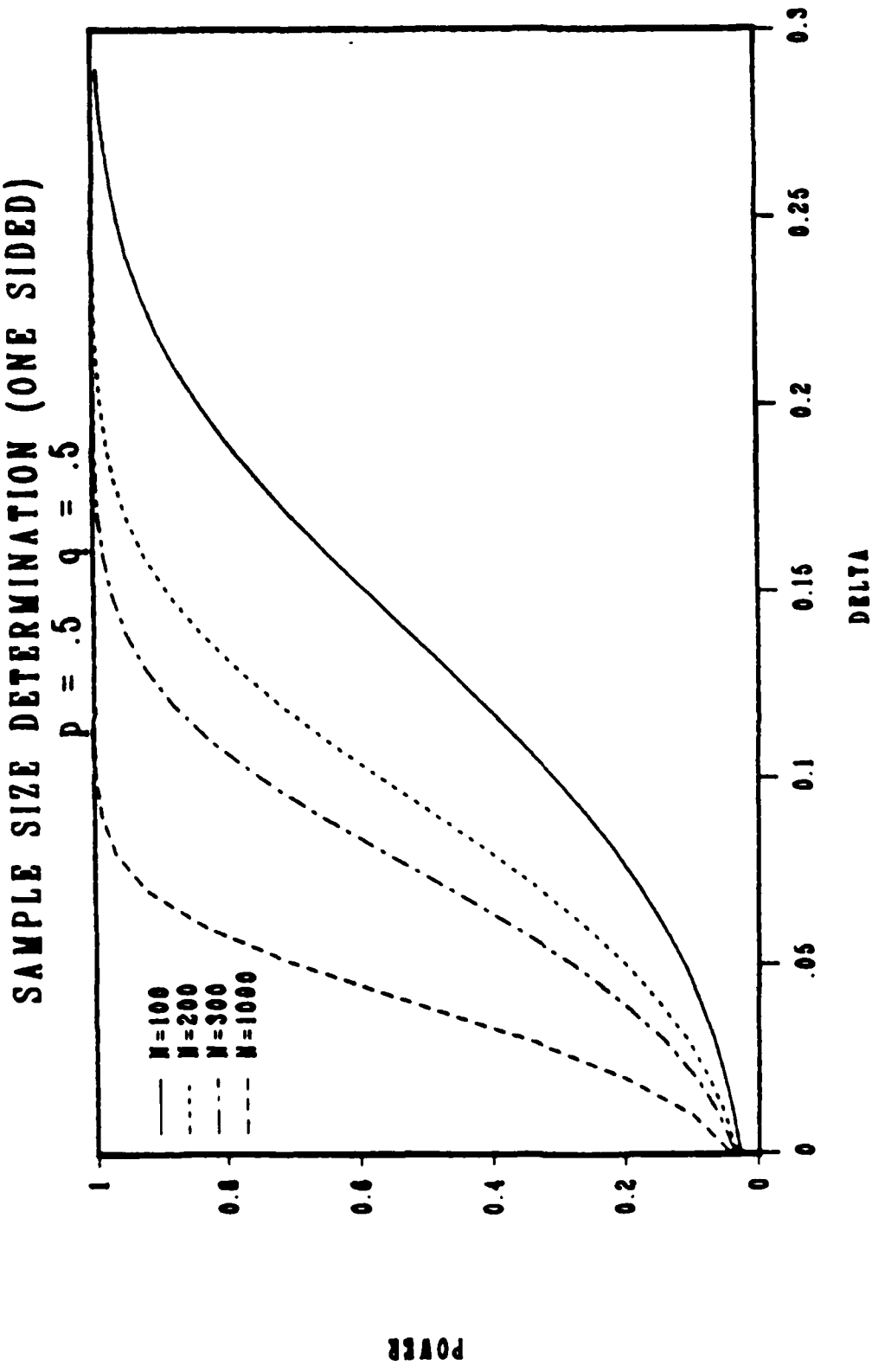


Figure 7. Operating curve used in selecting animal model.

IV. STUDY LOGISTICS

Once efforts to identify a suitable exposure facility, exposure dose, cage design, watering system design, animal model, animal number, and exposure duration were completed, efforts were devoted to defining appropriate logistics for conducting a study of RFR effects on cell growth and differentiation. These efforts were undertaken in collaboration with Dr. Kovatch and were initially concerned with the need for SOPs.

Standard Operating Procedures

To provide guidelines for laboratory work and a means by which quality assurance personnel can measure quality, all important procedures, processes, and tasks to be performed must be clearly defined and documented. The most common method for doing this is to establish and maintain a complete set of SOPs that clearly and concisely describe these important laboratory procedures, processes, and tasks. Standardization of these procedures, processes, and tasks will accomplish the following:

- Laboratory personnel will have written guidelines, eliminating problems with miscommunications and misinformation.
- Uniformity of procedures, and therefore uniformity of end item quality, will be assured.
- A written record of procedures will be available for review.
- A means of assessing the performance of procedures will be available for quality assurance audits.

Personnel from both Georgia Tech and PAI are fully aware of the importance of SOPs, and began during this program to (1) determine the applicability of their existing procedures to a study of RFR effects on cell growth and differentiation, and (2) develop additional procedures where needed. PAI already has in place SOPs for all aspects of pathology from necropsy to report formulation and final archiving/transport of pathology materials. Further, PAI has extensive experience in the conduct of Good Laboratory Practice compliance studies, having completed more than 500 rodent bioassay studies in the past 4 years under Good Laboratory Practice requirements. For example, Figure 8 shows a sample Toxicology SOP used by PAI QA personnel during their audit activities. Figure 9 is a sample Facility Inspection Checklist that serves as a guide to the development of appropriate SOPs. A list of PAI SOPs for pathology services is presented in Figure 10.

Pathology Associates, Inc. has assumed the responsibility of assuring that all SOPs needed at Georgia Tech will be in place before receipt of the mice. Procedures established, or in the process of being established, include

- animal room preparation,
- animal care and husbandry,

Within the laboratory's SOP, are at least the following items addressed?

Yes	No	
		Sanitization of animal rooms before the receipt of animals
		Receipt and examination of animals
		Quarantine, health evaluation, and release of animals for study
		Randomization of animals
		Identification of animals
		Receipt and storage of feed
		Receipt and storage of bedding
		Pest control procedures
		Rack, cage, and bedding change
		Watering system
		Operation and maintenance of cage and rack washers
		Sanitization of racks, cages, feeders, and watering system
		Sanitization of test facility and test rooms during the study
		Feeding and change of feeders
		Rack and cage rotation
		Observation of animals: daily AM and PM check, detailed clinical observation
		Procedures for disease control and for prevention of microbial spread in the test facility
		Environmental conditions of test rooms
		Handling of dead and moribund animals and criteria for moribund sacrifice
		Criteria for isolation of animals on test
		Gavage procedure
		Skin paint procedure
		Weighing of animals
		Evaluation feed and/or water consumption
		Terminal sacrifice of test animals

Figure 8. Checklist for toxicology SOPs.

Are SOPs present for:

YES	NO	
		1. Waste disposal
		2. Entry and exit from the testing area (including traffic patterns of dose prep facility and animal handling and testing room)
		3. Spill clean-up, accident and emergency response
		4. Employee training
		5. Respirator protection and fit
		6. Safety glasses
		7. Personal protective clothing
		8. Ventilation system maintenance
		9. Storage and transportation of test materials
		10. Use of radio-labeled materials (if applicable)
		11. Dose preparation
		12. Medical surveillance
		13. General housekeeping practices (eg.,cleaning of exposed pipes and light fixtures)
		14. Methods, materials, and schedules to be used in the routine inspection, cleaning, maintenance, testing and calibration of equipment.

COMMENTS:

Figure 9. Sample facility inspection SOP.

SOP Number	Title	Status (date of approval)
001	SOP for Necropsy	Completed (11/83)
002	SOP for Tissue Trimming	Revised (02/86)
003	SOP for Tissue Processing	Revised (06/84)
004	SOP for Tissue Processing-Fisher	Revised (12/85)
005	SOP for Embedding	Revised (06/84)
006	SOP for Microtomy	Revised (12/85)
007	SOP for Staining	Completed (11/83)
008	SOP for Coverslipping and Labeling	Revised (04/84)
009	SOP for Quality Control	Completed (01/86)
010	SOP for Archives	Revised (04/86)
011		Retired
012	SOP for Decalcification of Bone	Revised (02/86)

Figure 10. PAI SOPs for pathology service (1 of 3).

SOP number	Title	Status (date of approval)
013	SOP for Rodent Necropsy	Completed (01/86)
014		Retired
015	SOP for Canine Necropsy	Completed (04/84)
016	SOP for Data Transmission	Revised (04/84)
017	SOP for Equipment Operation & Maintenance	Revised (04/86)
018	SOP for Rodent Necropsy (MRI)	Revised (04/86)
019	SOP for Data Security	Completed (07/84)
020	SOP for Automatic Staining	Revised (04/86)
021	SOP for Pathology Material Inventories, Animal Identification, and Corrections	Completed (03/85)
022	SOP for LABCAT for Histopathology	Completed (03/85)
023	SOP for Block Sealing	Completed (10/85)
024	SOP for Assignment of Histology Numbers	Revised (11/85)
025	SOP for Transfer of Material to Laboratory from Archive	Completed (02/86)
026	SOP for Gross Specimen Photography	Completed (12/85)
027	SOP for Transportation of Pathology Specimens and Materials	Revised (01/86)

Figure 10. PAI SOPs for pathology service (2 of 3).

SOP number	Title	Status (date of approval)
028	SOP for Tissue Begging	Completed (03/86)
029	SOP for Use of Electronic Balance	Completed (04/86)
030	SOP for TDMS Data Entry and Quality Control	Revised (04/86)
031	SOP for Completion of PEIS Forms	Complete (04/86)
032	SOP for Organ Weighing	Complete (04/86)
033	SOP for Quality Control Sheet	Complete (05/86)

Figure 10. PAI SOPs for pathology service (3 of 3).

- animal identification,
- receipt, identification, storage, handling, and mixing of study articles
- clinical observations,
- test system observations,
- maintenance and calibration of equipment,
- transfer, proper placement, and identification of animals, and
- data storage, handling, and retrieval.

Logistics

Approximately 3 months after contract award, 450 female C3H/HeJ mice will be ordered from a suitable commercial vendor. Upon arrival at Georgia Tech, the mice will be quarantined in the test facility for a period of 2 weeks. At the beginning of this quarantine, an identification scheme involving toe clips and ear punches will be implemented, and 10 mice will be randomly selected to serve as sentinel animals. These animals will be euthanatized and necropsied as part of the necropsy training program and as the initial part of the quality control program. The training program will involve Dr. Kovatch instructing Georgia Tech personnel in the procedures necessary to conduct a complete necropsy. Georgia Tech personnel with M.D. degrees will be available for this training. The quality control part of the necropsy will characterize the health status of the animals as received from the vendor. Specimens for virology, bacteriology, and parasitology examination will be taken at this time. Results of this necropsy will provide a baseline against which results of subsequent necropsies of sentinel animals will be compared.

The time at which the quarantine is completed will be designated as Time Point 0, and RFR exposure will be initiated at this time. One day before Time Point 0, Dr. Kovatch will again visit Georgia Tech to conduct the next part of the disease surveillance/quality control program. Georgia Tech personnel will be further instructed as another 10 sentinel animals are necropsied. Specimens for serology, bacteriology, and parasitology examinations will be taken, after which Georgia Tech personnel responsible for unscheduled necropsies will have been adequately trained.

At Time Point 0, animal locations on the Radiation and Control Room waveguides will be randomized. The exposure field will then be energized at a level corresponding to a SAR of 0.32 W/kg.

For the 6 months following Time Point 0, Georgia Tech personnel will operate the exposure facility such that RFR is provided 20 hours daily 7 days each week. Standard Operating Procedures defining facility maintenance and operation, daily care and observation of animals, unscheduled necropsies, data records, etc. will be meticulously implemented.

At Time Point 6 months, Dr. Kovatch will return to Georgia Tech to supervise the third scheduled necropsy of 10 sentinel animals. Samples will again be taken for serology, bacteriololgy, and parasitology examinations. He will also review records of animal care, data collection, and facility operation. Tissue and necropsy records resulting from any unscheduled deaths will be collected. Tissues from these deaths will be processed, evaluated, and reported on. A critical phase inspection of procedures and records will be conducted to determine compliance with Good Laboratory Practice requirements.

At Time Point 12 months, the activities of Georgia Tech and PAI personnel at Time Point 6 months will be repeated. Approximately 40 animals will have died at this time point. Tissues from these animals (plus 10 more sentinel animals) will be processed and evaluated, and Dr. Kovatch will formally discuss the evaluation results with Georgia Tech and USAF personnel.

The RFR exposure will be terminated at Time Point 18 months, at which time an estimated 308 exposed, sham-exposed, and sentinel animals will remain. A PAI team composed of Dr. Kovatch, 5 prosectors, and 1 necropsy technical assistant will travel to Georgia Tech to conduct the large-scale scheduled necropsy. This team will have available supplies that will have been transported by van from PAI. All remaining animals will be euthanatized by cervical dislocation. The necropsy will require approximately 5 days to complete; therefore, each prosector will necropsy an average of 12 mice daily. These necropsies will be conducted in the Surgery Laboratory located in room 24 of the Baker Building on the Gerogia Tech main campus. This laboratory opens onto the same hallway as the RFR Exposure Facility, and is equipped with an exhaust to outside air, ample laboratory bench area, surgical tools and instruments, an autoclave, a refrigerator, etc. Hooded work stations connected to the external exhaust will be provided as necessary for personnel protection during the necropsies.

This scheduled necropsy will be monitored as a critical phase in the study, and all files and records will be reviewed.

Following completion of the scheduled necropsy, all tissue specimens will be inventoried, packed for safe transport, and returned to PAI for processing.

Four months after the scheduled necropsy, the draft version of the final Pathology Report will be submitted to Georgia Tech by PAI. This report, plus the draft version of the Engineering Report, will be submitted to the U.S. Air Force. A Quality Assurance Certification Statement will accompany the Pathology Report. During the U.S. Air Force review of the two draft Final Reports, Georgia Tech and PAI personnel will travel to Brooks AFB to formally present an overview of the study. Following U.S. Air Force approval of the draft reports, final Engineering and Pathology Reports will be published.

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APPENDIX

PROPOSED PROTOCOL FOR CHRONIC IN VIVO STUDY OF RADIOFREQUENCY RADIATION BIOEFFECTS ON MAMMALIAN CELL GROWTH AND DIFFERENTIATION

PROPOSED PROTOCOL FOR CHRONIC IN VIVO STUDY OF RADIOFREQUENCY RADIATION BIOEFFECTS ON MAMMALIAN CELL GROWTH AND DIFFERENTIATION

The study conducted under Task 27 of Contract No. F33615-83-D-0601 clearly established the feasibility of using a multi-animal radiation facility and a large population of experimental animals to investigate the bioeffects of chronic radiofrequency radiation exposure on the growth and differentiation of rapidly growing mammalian cells in vivo. The protocol provided in this appendix presents detailed descriptions of proposed methods and procedures for undertaking this study. In the following paragraphs, the proposed protocol is presented for the

- animal model,
- exposure field,
- animal housing,
- animal watering system,
- dose level,
- necropsy and histopathology protocols,
- pathology quality assurance procedures,
- standard operating procedures,
- study logistics, and
- study documentation.

A. ANIMAL MODEL

In Section IV, we noted that factors influencing the selection of an appropriate animal model included the results of a previous USAF-sponsored bioeffects study [2], another bioeffects study by Szmigielski et al. [6], and the desire to assure statistically significant results. The previous USAF-sponsored bioeffects study indicated an apparent increased incidence of malignant neoplasms in exposed versus sham-exposed male rats, but no single tissue or organ system developed a significant increase in benign or malignant neoplasms. The study by Szmigielski et al. raised the concern that long-term RFR exposure may affect mammary tissues in female mice. Although direct correlations between these two studies could not be made, they generated a strong interest in RFR effects on mammary cell growth and differentiation in vivo.

The animal model proposed for the follow-on bioeffects study is the murine mammary tumor virus (MuMTV) C3H/HeJ mouse. Significant amounts of reference data exist on this particular substrain of mouse. For example, mammary tumors in the C3H mouse are among the most frequently studied spontaneously occurring neoplasms, and the incidence and time of occurrence in the C3H mouse are well documented in the literature. Also, the animal size permits 400 mice (200

exposed, 200 sham exposed) to be used, thereby providing a sample size that assures statistical significance for the resulting data. Upon receipt from the vendor, the mice will be quarantined for 2 weeks and then entered into the exposure schedule at approximately 5 weeks of age. The exposure schedule will be terminated after 18 months, with animals that live to term being approximately 77 weeks old. The incidence of mammary tumors in these mice will then exceed 50 %.

B. EXPOSURE FIELD

We proposed that the exposure field be generated by an existing radiation facility designed, constructed, and evaluated under USAF sponsorship and used during previous chronic studies of RFR bioeffects [8,9]. This facility is located in room 19 (basement level) of the Baker Building on the main Georgia Tech campus. An overall description of the 8 rooms comprising this facility is provided in Section II.

The exposure field in this facility is generated by four sets of circular, parallel plates that are stacked one above the other in the Radiation Room. Each set of plates functions as an open-ended waveguide to generate an RFR exposure field, the characteristics of which are determined primarily by the separation distance between the plates and the type of antenna used to feed the plates. The 45.72-cm (18 in.) separation distance used in the existing facility (1) assures that only the lowest-order TE-mode propagates between the plates, and (2) defines the frequency bandwidth to be approximately 420 to 450 MHz. The feed antennas are slotted cylinders positioned vertically at the center of each set of plates.

The combination of separation distance and feed antennas assures that a horizontally polarized exposure field with an essentially uniform amplitude distribution in the azimuth plane is generated around the periphery of each set of plates. In the vertical plane, this field has a cosine distribution, so the power distribution is cosine squared. Therefore, placement of caged animals midway between each set of plates and at the plate periphery provides horizontally polarized exposure to an essentially uniform field with the maximum power density. We proposed this exposure field and animal placement for the chronic in vivo study of RFR bioeffects on mammalian cell growth and differentiation.

Since the open-ended waveguides in the Radiation Facility have diameters of 3.66 m (12 ft), their circumference is 1148.08 cm (452 in.). The cage dimensions, the intercage separation distance, and the circumference determine the number of cages that can be conveniently located in the Radiation and Control Rooms during a bioeffects study. For example, if the cage width and intercage separation distance were both maintained at 12.7 cm (5 in.), 45 cages could be positioned on the plates. As the width and/or separation distance are varied, the number of cages that can be positioned on the open-ended waveguides increases or decreases correspondingly.

C. ANIMAL HOUSING

The cages designed, constructed, and evaluated as described in Section III are proposed for use during the forthcoming study. The sides of these cages are constructed of Plexiglas material with floor and top materials being glass rods

arranged in a Plexiglas frame. Plexiglas is essentially transparent to electromagnetic waves at 435 MHz. Plexiglas also permits easy visual observation of the animals and can be conveniently washed in commercial cage washers. The cages provide a floor area of 161.3 cm^2 (25 $in.^2$) (12.7 cm (5 in.) x 12.7 cm (5 in.)), which is 67% larger than required by the National Institutes of Health (NIH) guidelines on minimum animal housing requirements [1]. The cage height is 12.7 cm (5 in.). The glass rods in the floor are spaced to provide easy disposal of feces and urine and to adequately support the mice. The combination of glass rods in both the floor and top assures adequate ventilation for the cage.

Food is delivered to the animal by means of a food hopper on the cage side. Animal access to this food is through a grate composed of vertical glass rods built into the cage side. The glass rods are spaced to permit ad libitum feeding. The hopper will hold enough food to last approximately 1 week.

D. ANIMAL WATERING SYSTEM

As described in Section III, a watering system that will reliably serve 400 mice located on the circular, parallel-plate waveguides in the Radiation and Control Rooms was designed and evaluated. We proposed that this watering system be used during the following study of chronic RFR bioeffects on mammalian cell growth and differentiation in vivo.

The proposed watering system consists of a distribution subsystem connected to drinking positions which are mounted on the individual cages. The distribution subsystem is composed of (1) water reservoirs mounted on top of the circular, parallel-plate waveguide stacks, (2) Norprene thermoplastic elastomer tubing routed around the periphery of the circular waveguides, and (3) various plastic couplings, valves, adapters, etc. The water reservoirs contain approximately 15.2 L (4 gal) of water and provide a valve-controlled output to the distribution subsystem. The Norprene tubing and plastic fittings connect the reservoirs with the cage-mounted drinking positions. The drinking positions are all-plastic devices that are glued to the cage end and are referred to as Hartcups. Each Hartcup has a trip-lever that is nudged by the mouse to raise a plunger that controls a polymer seal in the water distribution subsystem. The small quantity of water released from the polymer seal collects on the bottom surface of the Hartcup and is drunk from there by the mouse.

All components of this watering system are constructed of plastic materials that possess electrical properties that render them essentially transparent to RFR fields at 435 MHz. The water column that is necessarily a part of the distribution subsystem is routed either against the metal plates where the electric field vector is zero, or vertical so coupling of the horizontally polarized electric field vector is minimal. The water reservoirs are mounted on top of the waveguides and are, therefore, completely out of the exposure field.

E. DOSE LEVEL

Section III describes the theoretical and experimental dosimetry determinations that were made for a 1.0 mW/cm^2 exposure field. These determinations indicated that, theoretically, an absorbed dose of approximately 0.1 W/kg should be expected for a medium-sized mouse and an exposure frequency of 435 MHz. Experimentally, dosimetry measurements using medium-sized mouse

carcasses exposed to 435-MHz RFR in the existing facility yielded average absorbed dose levels of 0.32 W/kg.

For the follow-on study of RFR bioeffects on cell growth and differentiation, we proposed that the transmitter feeding the circular, parallel plate waveguides be adjusted to provide a 1.0 mW/cm^2 exposure field power density. The experimental dosimetry measurements showed that this will yield an absorbed dose of 0.32 W/kg.

F. NECROPSY AND HISTOPATHOLOGY PROTOCOLS

The proposed protocol for necropsy and histopathology is presented in chronological order in this Subsection. Therefore, the necropsy protocol is described first, then all aspects of the histology protocol are presented, and finally, tissue evaluation and data entry are described.

1. Necropsy Protocol

The research team proposed for the follow-on study has a full appreciation of the critical importance of necropsy. In the chain of events necessary for proper pathologic evaluation, the necropsy task stands out as one which cannot be duplicated if not done in a thorough and accurate manner the first time. Complete necropsy procedures ensure proper accountability of protocol-specified tissues for later tissue preparation and evaluation procedures. A 100% accountability of lesions and tissues is not only a necessity for later histologic evaluation, but the complete correlation of gross lesions with microscopic findings is a Good Laboratory Practice requirement.

Pathology Associates, Inc. will take full advantage of their corporate experience and of the specific experience of pathologist Dr. Robert Kovatch in the conduct and supervision of large-scale rodent necropsies at Georgia Tech. Dr. Kovatch has more than 20-years experience in rodent pathology and is recognized and respected as an authority in rodent toxicologic pathology. He will be supported by an experienced technical staff that will conduct the terminal scheduled necropsy.

1.1 General technical approach to scheduled necropsy

We proposed that a complete necropsy be performed on all exposed and sham-exposed animals for the scheduled terminal euthanasia at the conclusions of the 18-month RFR bioeffects study. A complete necropsy is defined as external examination, including body orifices, and examination and fixation of all protocol-specified tissues as listed in Figure A-1. All scheduled necropsies will be initiated within 5 minutes after the animal is killed.

All aspects of this scheduled necropsy will be performed in the presence, and under the supervision, of Dr. Kovatch. Dr. Kovatch will ensure that the necropsy procedure is performed according to Standard Operating Procedures (SOPs) and the specific protocol, to include animal identification and recording of gross lesions.

Gross lesions and tissue masses (and regional lymph nodes, if possible)
Mandibular and mesenteric lymph nodes
Salivary gland
Femur, including marrow
Thyroid
Parathyroids
Small intestine (duodenum, jejunum, and ileum)
Large intestine (cecum, colon, and rectum)
Liver
Gallbladder (mouse only)
Prostate
Testes/epididymis/ seminal vesicle
Ovaries
Lungs and mainstem bronchi
Nasal cavity and nasal turbinates (3)
Preputial or clitoral glands (paired) - rats only
Heart
Esophagus
Stomach (to include forestomach and glandular stomach)
Uterus
Brain (three sections, including frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons)
Thymus
Trachea
Pancreas
Spleen
Kidneys
Adrenals
Urinary bladder
Pituitary
Spinal cord and Sciatic nerve (if neurological signs were present)
Eyes (if grossly abnormal)
Mammary gland (to include surface skin)
Pharynx (if grossly abnormal)
Skin

Figure A-1. List of tissues for complete histopathologic evaluation.

1.1.1 Identification, verification, and accountability

The critically important points in the necropsy procedure lie in the following areas: (1) animal identification, (2) tissue accountability, (3) lesion recognition and accountability, (4) recording of gross findings and required entries on the Individual Animal Necropsy Record (IANR), (5) proper trimming of the wet specimens, and (6) tissue fixation.

Regarding animal identification, before euthanasia each animal's unique identification number will be verified and matched to the number on the IANR form and specimen bottle. To ensure maximum tissue accountability, compartmentalized, clear plastic necropsy trays, each of which overlies a template indicating required protocol-specified tissues, will be used. Also, each tray will be conveniently divided into subgroups for maximum efficiency in tissue harvesting and verification. Within each compartment, a small amount of fixative will be placed to prevent tissue drying and to initiate immediate tissue fixation after harvesting. The use of these trays will not only serve as a required tissue checklist for prosectors, but will also enable the supervising pathologist to visually inspect each required tissue for lesions and accountability before its placement in the container of fixative (eosin tinged 10% neutral buffered formalin).

When the prosector has finished an animal, the tray containing the tissues and carcass, the prelabeled fixative container, and the completed IANR will be brought to the pathologist's station centralized within the necropsy room. The pathologist will first verify that the identification number on the animal, IANR, and container are identical. This step will have already been done independently by the necropsy assistant when providing the prosector with the animal, necropsy tray, container, and necropsy form, and subsequently by the prosector before initiating dissection.

After inspecting the IANR for appropriate entries, the pathologist will systematically examine each tissue within the compartments of the tray as it is removed and placed in the fixative container. This important step will enable the pathologist to ensure a maximum accountability of tissues, to verify all gross findings noted by the prosector, and to identify any additional changes deemed significant. Finally, before signing the IANR, the pathologist will ensure that all required entries on the necropsy form are present and correct, including disposition codes, correction notations, prosector's signature, and date.

1.2 Specific Necropsy Procedures

For the scheduled gross necropsy conducted at the 18-month terminal euthanasia and for all unscheduled (moribund euthanasia and spontaneous deaths) necropsies, the following specific necropsy procedures will be followed:

- Under the supervision of Dr. Kovatch, scheduled necropsies will be initiated within 5 minutes after an animal is killed.

- All tissues and/or organs will be examined in situ; then dissected from the carcass in the manner specified below; reexamined, including cut surfaces; and fixed in 10% neutral buffered formalin. Tissues saved for histopathology will be fixed at a thickness not to exceed

0.5 cm except as stated below. Ears and/or feet that have been used in any way for animal identification during the in-life phase of the study will be saved in formalin.

- The number of grossly visible nodules (tumors) of similar morphological appearance will be recorded up to five per organ. After five nodules have been counted, "greater than five" will be recorded.

- The trachea and lungs will be perfused by introducing 10% buffered formalin (approximately 1-2 mL for mice) into the trachea until the lungs are completely filled to normal inspiratory volume.

- The calvaria will be removed for examination of the brain and pituitary. The brain will be removed for fixation and pituitary left in situ for fixation but closely observed for abnormalities. The nasal bones will not be removed. The head will be retained for decalcification. Further, formalin will be back-flushed from the larynx through the nares to assure proper fixation of the nasal cavity structures.

- Distended urinary bladders will be fixed "as is." Contracted, empty bladders will be partially distended with formalin. Urinary bladders will be opened and examined after fixation at trimming. Care will be taken to insert the needle into the lumen of the bladder to avoid artifacts caused by insertion into the bladder wall.

- The liver will be sliced to ensure fixation. The kidneys will be bisected, and the cut surfaces will be examined before fixation. The left kidney will be bisected longitudinally and the right kidney transversely.

- The mandible will be removed to allow visualization of the oral cavity to include the tongue and pharynx.

- The pelvis will be split and the entire gastrointestinal tract, including the anus, removed. The entire gastrointestinal tract will be perfused with formalin, including the stomach, to ensure immediate fixation of the mucosal surface. The stomach will be opened later and examined during tissue trimming. The entire intestinal tract will be opened and examined leaving 2-3 cm segments of duodenum, jejunum, ileum, colon, cecum, and rectum intact for cross sections.

- Multiple representative portions of large or variable tissue masses or areas of tissue change, including surrounding unaffected tissues, will be fixed. Very small masses may be fixed in their entirety.

- Several thoraco-lumbar vertebrae will be fixed with the spinal cord in situ.

- A section of the heart will be sliced from the base through the apex so that all four chambers are visualized.

- The oral cavity, pharynx, and larynx will be carefully examined grossly. If any abnormalities are noted, the tissues will be examined microscopically.

During the conduct of the necropsy, all gross lesions will be recorded using the terminology in the Toxicology Data Management System (TDMS) Pathology Code Table (PCT) inclusive of morphologic lesion; anatomic site; quantity or size in milliliters, millimeters, or number; shape; color; and consistency. Each gross observation will ultimately be correlated with a microscopic evaluation.

Animals will be individually weighed at necropsy. Carcasses will be placed in storage bags and fixed in 10% buffered formalin.

Color photographs showing scale and identification will be taken on a selective basis. The photographs will be submitted to the U.S. Air Force with the original copy of the pathology narrative. Each photograph will be identified with the contract number, dose level, animal number, sex, and histologic diagnosis.

1.3 Unscheduled Necropsies

All necropsies for spontaneous deaths or other unscheduled euthanasia will be performed as soon after death as possible. At no time will animals be frozen, and every attempt will be made to refrigerate animals for no longer than 4 hours before the necropsy being performed. At necropsy, an effort will be made to establish the cause of death. Dr. Kovatch will conduct training courses during three separate site visits to assure that Georgia Tech personnel practice acceptable techniques in the harvest and fixation of protocol-specified tissues. All interim deaths will be subjected to the same thorough necropsy as scheduled euthanasia.

2. Histology Protocol

For the proposed 18-month RFR exposure all aspects of tissue preparation will be performed in accordance with PAI SOPs that meet the requirements of the EPA and FDA Good Laboratory Practices, and of the specific protocol proposed herein.

The critical histology procedures are briefly described later as they will apply step-by-step to tissue being processed in the PAI laboratory. This description incorporates portions of PAI's SOPs as well as specific processing instructions that are addressed in the following paragraphs. We emphasized that all functions in slide preparation are covered by SOPs. Also, the IANR and a copy of the PAI Quality Control Sheet routinely accompany the tissues throughout the laboratory. These documents are available to the technicians for entry of additional findings and comments, as appropriate.

2.1 Tissue Trimming

Tissue trimming will be supervised by Dr. Kovatch and the PAI Laboratory Supervisor. Before trimming is initiated, the technician will review the information on the IANR for each animal. All lesions identified at gross necropsy will be trimmed or, if not observed in the fixed tissue, appropriate

notations made on the IANR. All additional gross changes observed in the fixed specimens will be recorded on the IANR using the appropriate TDMS PCT terminology. All missing tissues will be recorded.

A predetermined trimming/embedding scheme will be prepared for each study protocol. The placement scheme is designed to afford the best opportunity to consistently obtain adequate sections of all protocol-specified tissues. The specific scheme will be attached to the trimming hoods for easy reference.

Tissue will be trimmed to a maximum thickness of 0.3 cm for processing. The trimmed specimens will be placed in white plastic Tissue-Tek II cassettes. Each of the cassettes will be labeled with the correct histology number. This label will be written on the plastic cassette housing using a #2 hard lead pencil.

Tissues that require decalcification (bone, calcified lesions, etc.) will be identified, labeled, and placed in formic acid-sodium citrate solution. This technique (Armed Forces Institute of Pathology (AFIP) Staining Manual, page 8) gives better staining results than the commercially available preparations since formic acid-sodium citrate is less harsh on the cellular properties. Pathology Associates, Inc. has found this method to be particularly effective in cases of overexposure of tissue after decalcification is completed since there is little compromise of staining qualities.

In accordance with SOPs and modifications, all tissues required for complete histopathology examination will be trimmed, including gross lesions from all exposed and sham-exposed animals. The tissues will be trimmed within a period not less than 48 hours nor greater than 12 weeks following necropsy. For this study, we projected that the tissues will be completely trimmed within 4 weeks of receipt of the formalin-fixed wet tissues.

In accordance with PAI SOPs, the following specific methods of tissue trimming will be followed:

- Multiple portions of tumors, masses, foci, and areas of tissue change will be submitted if these are large or variable in appearance. Surrounding, normal tissue will be included if possible with the lesions.

- Parenchymal organs (e.g., liver), will be free of adjacent tissues and trimmed to allow the largest cross-section surface area possible for examination. For liver and lung, one section of each nodule (tumor) will be prepared, up to five tumors for each organ. The five largest tumors or lesions will be sectioned if more than five are observed. Adjacent normal tissue will be included along with the lesion. At least two sections of normal liver will be prepared including sections through left and right lobes. One section of liver will include the adjacent common bile duct or gall bladder.

- Mid-longitudinal section (left kidney) and cross section (right kidney) through the entire cortex and medulla of each kidney will be submitted.

- Three cross sections of the brain will include (a) frontal cortex and basal ganglia, (b) parietal cortex and thalamus, and (c) cerebellum and pons. If any lesions are observed after sectioning, they will be noted on the IANR.

- The entire coronal (perpendicular to a sagittal plane and parallel to the long axis of the body) section of both right and left lungs including mainstem bronchi will be submitted.

- Hollow organs will be trimmed and blocked to allow a cross-section slide from mucosa to serosa.

- Following fixation, the trachea will be opened to the level of the hilus and grossly examined. Any tumors or abnormalities in the trachea will be examined microscopically.

- Following fixation, the pituitary will be carefully removed and trimmed to allow a coronal section.

- After decalcification of the head, three separate sections will be taken at (1) the level of the incisor teeth, (2) midway between incisors and first molar, and (3) middle of second molar (olfactory region). The remainder of the nasal cavity and turbinates will be carefully examined for gross lesions at this time. The gross lesions will be recorded on the IANRs.

Tissues will be trimmed to a maximum thickness of 0.3 cm for processing. Small (less than 0.3 cm) endocrine organs, lymph nodes, and tissue masses will be submitted intact. One cross section will be prepared from each thyroid (two per animal), adrenal (two per animal), pituitary (one per animal), and ovary (two per animal).

All residual tissues from all animals will be double bagged in 10% buffered formalin following trimming and identified appropriately.

When the tissue trimming for each accession number is completed, the total number of cassettes will be tabulated and the number entered on the Quality Control Sheet. At this time, the technician will check all cassette labels against those on the IANR. The cassettes will be placed in baskets with a 70% aqueous solution of ethyl alcohol and placed on a shelf to await tissue processing. The technician performing the trimming will record the date and initial the IANR form and Quality Control sheet.

2.2 Tissue Processing

Fully automated processing units (Fisher 266MP and 166MP) will be used in the PAI laboratory. The standard processing schedule will be as specified in the SOPs. Temperature readings will be documented at least each working day to assure that proper temperatures of the paraffin units are maintained. Temperature data will be maintained in a log book kept in the processing section. Reagents used in processing will be checked and upgraded daily with a change of all reagents weekly or more frequently depending on amount of use. All reagent changes and dates will be recorded and initialed on the Processor Record Form.

Once processing is completed, the processed cassettes will be removed and taken to the infiltration/embedding station where they will be placed in vacuum infiltrators for 10 - 15 minutes to further facilitate paraffin infiltration.

The technician responsible for tissue processing will date and initial the processing/embedding log, the Quality Control Sheet, and the comment section of the IANR.

2.3 Tissue Embedding

The tissue embedding scheme used will be as determined by the specific protocol and by PAI SOPs. The embedding scheme for this particular study will be posted on the wall in the embedding section for easy reference.

Before actual embedding, the processed cassettes will be placed in the vacuum infiltrators for a minimum of 15 minutes. The temperature of the paraffin in the infiltration units will be checked and recorded daily and the paraffin will be exchanged according to the SOP.

Following infiltration, all tissues will be embedded in metal molds using a Tissue Tek II Center. The temperature of the paraffin reservoir will be checked and recorded daily.

After all tissues have been embedded and the blocks have cooled and been removed from the metal molds, the blocks will be arranged in order and checked for completeness (proper number of cassettes and proper labeling) against the IANR. The technician responsible for embedding will record the date and initial the IANR and Quality Control Sheet.

Following this check, the blocks will be placed in cabinets or specimen envelopes, the IANR and Quality Control Sheet will be attached, and the materials will be taken to the microtomy section.

2.4 Microtomy

Tissue sections will be prepared using AO (820 and 820S) rotary microtomes.

As the blocks are chilling, 2.54 cm x 7.62 cm (1 in. x 3 in.) glass microscope slides (unless another size is required) will be labeled appropriately with the project/histology accession number, in pencil, on the frosted end of the slide.

Sections will be cut routinely at 4-to-6 µm, and then mounted and dried on warmers. The temperature of the water bath used during sectioning will be checked and recorded daily, and the water will be changed according to SOPs.

After sectioning is completed, all penciled slide labels will be checked against the paraffin block labels with the necropsy form being used as the reference. The total number of slides prepared will be recorded and the necropsy form will be dated and initialed appropriately.

2.5 Staining, Labeling, and Coverslipping

After the slides have dried, they will be placed in staining racks, and stained routinely with hematoxylin and eosin (H&E) using a Gam Rad Stainomatic.

The reagents and chemicals used during staining will be checked and changed according to schedules established in the SOPs.

After staining is completed, the staining racks with slides will be placed in containers of xylene and taken to the coverslipping area. Slide coverslipping will be performed under specifically designed hoods as a protection against xylene and mounting medium fumes. The slides will then be placed in slide flats in accession number order and in preparation for labeling and drying.

A unique histology number will be assigned to each animal which is to receive histologic workup. At the time of assignment, this number will be entered in a permanent log and cross referenced. The histology number will appear on the label placed on the tissue block, on the slide, and on the label between the two bags containing the wet tissues. The format for labeling slides (tissue slides, blood smears, cytology smears, etc.) will be identical to that used by the National Toxicology Program (NTP). Each block and slide will be subnumbered from 1 to n to show which number that block/slide is for that animal.

Slide labels will be affixed to the microslides. Labels will be neatly printed or computer generated using information obtained from the necropsy form and as required by protocol. The labels will be placed on the frosted end of the slide. Completed slides will be allowed to dry and then placed in slide flats or boxes in preparation for delivery to the Quality Control Section.

The technician completing the labeling process, will again check the number of slides, labels, etc. for each accession number using the necropsy form as a reference.

2.6 Quality Control and Case Accountability

All PAI commercial laboratories have a separate Quality Control Section that is responsible for the review of slides, serves as a focal point for recut and retrim requests, and assembles the cases before delivery to the designated study pathologist. In all PAI facilities, this section is headed by a senior technician who is either American Society of Clinical Pathologists (ASCP) registered and/or has extensive experience in tissue recognition, slide artifact recognition, and large-volume case assembly. Although this section is formally recognized as being responsible for quality control, in all labs the quality control of each technician's individual work is the initial and perhaps most important step in a quality control program. Further, the "self-quality control" is augmented by structured quality control audit procedures performed by the senior technician in charge of each critical phase (trimming, processing, embedding, etc.) before the specimens leaving their area. The Laboratory Manager and Quality Control Technologist routinely monitor all aspects of tissue preparation to ensure compliance with SOPs.

As part of the quality control/tissue accountability procedures, a Quality Control Sheet accompanies each case as it progresses through the various critical phases of histologic processing. Information on this sheet is reviewed by each technician before further processing being initiated at their respective sections.

According to SOPs, completed cases will be delivered to the Quality Control Section. To maintain quality work within the Histology Laboratory, all slides will be checked both microscopically and macroscopically. The quality control procedures will be performed in accordance with PAI SOPs, will meet Good Laboratory Practice requirements, and will be constantly monitored by the PAI Quality Assurance Unit as well as by staff pathologists. Inputs from the latter will be especially relevant since, at PAI, the pathologists establish the guidelines and standards that determine the acceptable levels of product quality.

In accordance with SOPs, the procedures identified below will be routinely performed by the PAI Quality Control Section:

- Each slide will be checked for artifacts (mounting bubbles, knife marks, etc.) both grossly and microscopically.
- Using the necropsy form as a reference, all slide labels and the total slide numbers for each accession number will be checked.
- Paper label information will be checked against frosted end label information using the "mirror" procedure.
- Slides will be examined microscopically to assure that all tissues required by the specific protocol are present. In accordance with SOPs and Good Laboratory Practice requirements, there must be a 100% correlation of gross findings with a microscopic findings entry.
- Slides will be matched with the paraffin blocks as a further check.

Slides deficient in any of these areas will be returned along with the necessary materials to that section of the laboratory responsible for the deficiency. Returned materials will be accompanied by a Recut/Retrim form. During case assembly, the Quality Control Technologist will reference the computer-generated Archives Inventory Form ensuring that the tissues have been processed for each case originally identified as part of the study.

The Quality Control Technologist performing the quality control procedures will initial and date the IANR and Quality Control Sheet, deliver the slides to the pathologist for evaluation, and return the blocks to the Archives for storage.

The Quality Assurance Unit will closely monitor all aspects of the Quality Control Section and will conduct a "phase inspection" of the section each time histologic phases are examined. The specific approach to the phase inspection is presented in Subsection G.1.

3. Tissue Evaluation and Data Entry

For this proposed 18-month RFR exposure, Dr. Kovatch will perform a complete histopathologic evaluation, including gross lesions, for all animals that die before term. The histopathologic evaluation of tissues of all early deaths (found dead or moribund euthanasia) will be conducted immediately upon receipt and processing of the tissues.

Microscopic data will be entered using the Microscopic Observations Records with subsequent entry into the PAI LABCAT computer system.

3.1 Tissue Evaluation

During the systematic microscopic evaluation of the study tissues, histopathologic observations will be recorded on a Microscopic Observations form. Histopathologic diagnosis of all lesions will be entered under Organ and Diagnosis. For diagnosis and morphologic qualifiers, the NTP terminology as stated in the PCT will be used. All non-neoplastic lesions will be graded using a four grade system of minimal, mild, moderate, and marked.

3.2 Tissue Reevaluation

Following the initial review of the study tissues by the primary pathologist and receipt of the original summary tables, the PAI pathologist will evaluate the tables and terminology used. When necessary, a reevaluation of selected tissues will be conducted to include possible blind reads where critical evaluation of potential compound-related effects exist. If the re-evaluation is specifically intended to verify apparent, RFR-related changes that are quite subtle, the task will be conducted as a blind evaluation to prevent biases that could result in spurious trends in the data.

3.3 Data Entry

Following entry of each series of animals/exposure group, individual histopathology findings for each animal will be printed and there will be a 100% quality control check of the Micropath form findings with the computer-generated data, with a check to ensure that all gross lesions have an entry in column 22B (TGL) of the forms. This quality control check not only includes data verification by the original data entry clerk, but also a 100% validation by a second data entry clerk. Any discrepancies will be corrected before table generation. Other aspects of the generated data are discussed in subsection J.

All aspects of data entry will be conducted according to established SOPs and within the guidelines of EPA and FDA Good Laboratory Practices. The technical aspects of this task will be monitored by the PAI Quality Assurance Unit.

3.4 Data Analysis

Data from this experiment will be analyzed using two complementary techniques: analysis of the exposure and sham-exposure groups survival distribution functions, and analysis of variance (with treatment levels being RFR, time, etc.).

Survival curves will be generated for both exposure and sham-exposure groups (using product-limit and Kaplan-Meier estimates), and will be compared to one another using log rank and Wilcoxon statistics. Additional stratification within each group (comparing animals from different tiers of the waveguide) will be used as a check for systematic problems.

To determine whether the frequency of various organ lesions varies between exposure and sham-exposure groups, analysis of variance tables will be prepared from the necropsy data. Factors of the analysis include time, RFR, cause of death (natural or terminated) and organ type. Additionally, time series estimates of treatment effects will be computed to ensure that the autocorrelation of error in the experiment does not bias the significance of the ANOVA.

The analysis will be performed on the Georgia Tech IBM 4381 mainframe with Statistical Analysis System (SAS) software. Additional tests available on the BMDP and SPSS systems will be performed as needed.

G. PATHOLOGY QUALITY ASSURANCE PROCEDURES

Procedures for Quality Assurance auditing will be broken into two categories: (1) phase inspections, and (2) data audits. Phase inspections will consist of the actual observation of different phases being performed in the laboratory. Data audits will involve reviewing the raw data that are generated during, and at the completion of, the study.

1. Phase Inspections

Phase inspections will consist of the actual observation of the phases that occur within the scope of the pathology laboratory, and are typically termed as "critical phases" due to their potential effect on the study. These phases include

- necropsy,
- embedding,
- receiving of materials,
- trimming,
- staining, coverslipping/labeling,
- cutting, and
- packing and shipping inventory.

2. Data Auditing

Data auditing will occur during the phase inspections whenever material is received into the laboratory (incoming inventory of specimens and data sheets) as well as the data recording that occurs at each phase of the laboratory histologic preparation. These audits will cover the first three stages of a four-stage inspection that occurs for data. In particular, the first three stages will be made up of (a) at necropsy, (b) post necropsy, and (c) during processing. The final stage will be the post-processing stage which includes the Pathology Report, and data transcription (i.e., computer data entry), computer data generation, and shipping inventory.

The Pathology Report, computer data entry, and computer data generation will all be considered part of the report audit, as will a review of the individual animal data forms.

- Individual Animal Necropsy Records:
  IANRs for all animals will be reviewed for thoroughness, for conformance to Good Laboratory Practice guidelines, for conformance to SOPs, and for gross microscopic correlations. If errors are found, the forms will be returned to the study pathologist or appropriate laboratory personnel for review and correction of the discrepancy.

- Pathology Reports:
  Micropath forms will be compared to computerized Individual Animal Data Sheets to verify accuracy of data entry.

- Summary tables will be checked for correct header information and accountability of protocol-required tissues.

- Narrative portions of the report will be reviewed for accuracy and typographical errors. Besides the Quality Assurance review, the narrative will be independently reviewed by two additional pathologists to assure the most accurate presentation and interpretation of findings. All statements will be verified by reviewing the raw data. If any discrepancies are found or changes suggested, the report will be returned to the pathologist for review and/or correction.

- Residual Wet Tissues

- All residual tissues from a random 10% of the animals will be examined by an experienced histology technician for untrimmed nodules/masses that are potential tumors. If any missed lesions are found, they will be confirmed by the Quality Assurance pathologist, and then residual tissues from all animals will be checked for untrimmed lesions. Any potential tumors found as a result of the Quality Assurance wet tissue audit will be trimmed and slides prepared for evaluation by the study pathologist.

In all cases, the Quality Assurance Unit will report their findings directly to the General Manager of PAI. Corrections will be initiated by the General Manager and reported to the Quality Assurance Unit by memorandum.

H. STANDARD OPERATING PROCEDURES

All engineering and biological activities will be identified by and described in SOPs. The standardized procedure will eliminate misinformation transfer and miscommunications, assure that procedures are performed in a consistent manner, provide a written record of procedures for review, and make performance procedures available for quality assurance audits.

Both the FDA and EPA Good Laboratory Practice regulations/standards require that SOPs be available and maintained for all studies conducted in compliance with their requirements.

Pathology Associates, Inc., has SOPs for all aspects of pathology services from necropsy to report formulation, including final archiving/transport of pathology materials. In the engineering area, SOPs will be prepared for the maintenance and calibration of all exposure equipment and for test system observations. The PAI Quality Assurance Unit will conduct audits of this aspect of the study to assure that the engineering procedures are both available and maintained.

Regarding laboratory animal care, a full complement of SOPS will be prepared for all aspects of animal care, husbandry and in-life observations. PAI currently maintains SOPs covering these aspects as part of EPA on-site toxicology and carcinogenesis testing projects. These SOPs will be modified appropriately and used for operational aspects of this project. These Procedures will typically include the following activities:

- Sanitization of animal rooms before the receipt of animals
- Receipt and examination of animals
- Quarantine, health evaluation, and release of animals for study
- Randomization of animals
- Identification of animals
- Receipt and storage of feed
- Pest control procedures
- Watering System
- Operation and maintenance of cage washer
- Sanitization of cages and watering system
- Sanitization of test facility and test rooms during the study
- Procedures for disease control and for prevention of microbial spread in the test facility
- Environmental conditions of test rooms
- Handling of dead and moribund animals and criteria for moribund euthanasia
- Criteria for disposition of escaped animals
- Technician training
- Sentinel animal program
- Handling of emergencies
- Weighing of animals

- Observation of animals -- daily checks, detailed clinical observations, and recording of lesions

The SOP for observing the animals will specifically require an examination to identify all skin lesions. These lesions will be numbered and charted on a dorsal view of the mouse. This tumor map will become a permanent part of the animal's record and will accompany the study data through histopathology. Information such as the time of appearance, location, size, shape, progression from one type tumor to another, confluence of tumors, and time of regression will be recorded in the study data. The total number of tumors per animal, the number of tumor-bearing animals, the number of carcinomas per animal, and the number of mice with carcinomas will be summarized weekly.

The PAI Quality Assurance Unit will monitor critical phases of the in-life/laboratory animal care procedures to assure that all activities are conducted in compliance with Good Laboratory Practice requirements.

I. STUDY LOGISTICS

1. Study Team

The overall responsibility for directing and managing the study will be assigned to Mr. James Toler, Principal Research Engineer with the Georgia Tech Research Institute. Mr. Toler has directed and managed previous studies concerned with long-term, low-level effects of RFR in large rodent populations. He was also the Project Director of the program under which the proposed Radiation Facility was designed, constructed, and evaluated. Mr. Toler has 12 years of experience in studies concerned with the interaction of electromagnetic waves with biological systems. During this time, he has directed 21 research programs.

Mr. Toler will be assisted by Dr. Philip Kennedy and Mr. Stephen Bonasera. Dr. Kennedy, who holds both M.D. and Ph.D. degrees, will be responsible for the surgery associated with the unscheduled and scheduled necropsies before the terminal necropsy at Time Point 18 months. Dr. Kennedy's medical training as well as his continuing research programs result in his being ideally suited for this responsibility. Mr. Bonasera will be responsible for most of the day-to-day operation of the Radiation Facility and for the supervision of the Animal Caretaker. Mr. Bonasera is pursuing simultaneous advanced degrees in Electrical Engineering and Biology. He is thoroughly familiar with the proposed Radiation Facility and its operation since he worked during the latter part of the last chronic study in this facility. Mr. Bonasera has been trained in animal handling techniques at Emory University and is knowledgeable of requirements in the National Institutes of Health (NIH) guide for animal care. We anticipate that the Animal Caretaker will be Mr. Charles Vinson, who has worked for many years in animal care facilities at Emory University. Mr. Vinson was also the Animal Caretaker on a previous Georgia Tech study of long-term, low-level radiation effects in rats.

Dr. Robert Kovatch (whose qualifications were presented in Section III) will serve as the Principal Investigator from PAI. Dr. Kovatch has more than 20 years of experience in various aspects of experimental and diagnostic pathology. He is currently the Project Manager for pathology support for the NCI FCRF research program at Fort Detrick, Maryland. Dr. Kovatch has received

international acclaim as a rodent pathologist and has evaluated the effects of more than 100 chemicals in rodent bioassay test systems. He will have oversight responsibilities for all PAI project activities and scientific aspects of the study. This responsibility will include protocol design, results interpretation, preparation of reports, and training of Georgia Tech personnel. Dr. Kovatch will keep informed of project activities by maintaining communications with Mr. Toler and will, as a minimum, hold formal meetings with the Georgia Tech technical staff before each scheduled euthanasia.

Ms. Chris Sexsmith of PAI will serve as the Quality Assurance Officer responsible for ensuring, assessing, and documenting the quality of data produced. It is imperative that appropriate SOPs be in place and that the activities are monitored by the QAU to assure that data generated are of the highest quality and GLP-consistent. The Quality Assurance Unit's responsibilities include evaluation of personnel qualifications and training, facilities and equipment adequacy, inspection of critical phases, sampling procedures, and data validation. To accomplish this task, Ms. Sexsmith will familiarize all Georgia Tech personnel with basic Good Laboratory Practice concepts and the role of quality assurance. She will maintain the master time schedule; establish and maintain written Quality Assurance guidelines; ensure that the facilities, techniques, and equipment are adequate for successful completion of the study; and verify that all protocol-specified procedures are adequately performed by trained personnel. Ms. Sexsmith will also verify that all samples are properly selected, collected, and handled; and she will ensure that all equipment is operated, calibrated, maintained, and evaluated as described in the SOPs. She will verify that specified tissues are properly collected and identified at necropsy, that the information contained on the Tissue Processing Records is accurate and complete, and that any discrepancy noted is resolved. Finally, Ms. Sexsmith will perform in-depth data audits and assure that log books, data books, work sheets, and other hard copy information are properly filled out and signed by the responsible professional.

2. Preparatory Logistics

The first three months of the study will be devoted to preparations for receiving the mice and initiating the RFR. During this time, the scheme used to mark mouse cages will be implemented. This scheme will involve numbered placards that attach to the cages but can be removed during cage washing. Also, the additional thickness of Styrofoam (5.08 cm; 2 in.) needed to center the cages vertically between the waveguide plates will be purchased, cut to the proper radius, and positioned on the waveguides. The watering system for the waveguides in both the Radiation and Control Rooms will be installed. Materials and supplies necessary for proper care of the mice will be purchased. This care will include food, bedding, soil trays, deodorant paper, detergent, disinfectant, cleaning supplies, etc. The electronic balance will be interfaced with the computer so mice can be weighed ten times over a period of a few seconds. The average weight will then be automatically calculated and transferred into the computer along with the animal's identification number. Any SOPs that have not already been generated will be prepared. A record system consisting of separate files for each mouse will be developed. This system will provide entry points for data on weight, general appearance, health status, etc.

When all of these preparatory tasks are essentially complete, 450 female C3H/HeJ mice will be ordered from a suitable commercial vendor (Jackson

Laboratory, Bar Harbor, Maine). Upon receipt, 440 of the mice will be weighed, assigned numbers, and randomly transferred to individual cages that will be in place on the Radiation and Control Room waveguide structures. The assignment of animal numbers will be by means of toe clips and ear punches. The remaining ten mice (sentinel animals), which will have been randomly selected from the 450, will be euthanatized and necropsied as part of the necropsy training program. This training program will involve Dr. Kovatch instructing Georgia Tech personnel in the procedures necessary to conduct a complete necropsy. Georgia Tech personnel with M.D. degrees will be available for this training. Specimens for virology, bacteriology, and parasitology will be taken. Results of this necropsy will provide the baseline against which results of subsequent necropsies of sentinel animals will be compared. These results will also characterize the health status of the animals immediately upon receipt from the vendor and confirm the quality control status of the animals provided by the vendor.

When these tasks are completed, a 2-week quarantine period will begin. During this period, requirements in the SOPs will be followed with respect to daily observations and visual inspections of the animals, record keeping, weekly weighings, weekly cage washings, daily cleaning of the Radiation and Control Rooms, and changing of the soil trays.

The day/night cycle for the animals in both rooms will also be established during this quarantine period. The timer that controls lighting in the Radiation and Control Rooms will be adjusted such that a 12-hour-on/12-hour-off cycle is established. The "night" half of the cycle will extend from 12 noon through 12 midnight. This cycle will allow facility cleaning, animal observation, transmitter maintenance, etc. to be accomplished during normal work hours between approximately 8:00 a.m. and 12 noon each day.

Ms. Chris Sexsmith, PAI Director of Quality Assurance, will be at Georgia Tech during the initial part of this quarantine period and will review all existing SOPs with respect to the engineering, in-life, and pathology aspects of the study. She will inspect the facilities, examine necropsy training results, observe the necropsy of sentinel animals, and monitor routine animal care/husbandry practices to assure Good Laboratory Practice compliance. As an additional part of her responsibilities, Ms. Sexsmith will assist in the modification and/or preparation of SOPs to assure that they accurately reflect the manner in which tasks are actually performed and that all study aspects are in compliance with Good Laboratory Practice requirements. Following the site visit, Ms. Sexsmith will complete her initial Quality Assurance responsibilities by making any necessary corrections to SOPs and by recommending needed action items for Georgia Tech personnel that will assure Good Laboratory Practice compliance. Revisions recommended by Ms. Sexsmith will be incorporated into the applicable SOPs so correct procedures can be followed from the initiation of the study.

One day before initiation of RFR exposure, Dr. Kovatch and Ms. Sexsmith will return to Georgia Tech to conduct the next aspect of the disease surveillance/quality control program. Again, ten sentinel animals will be necropsied during which time the training of Georgia Tech personnel in necropsy techniques will continue. Specimens will be taken for serology, bacteriology, and parasitology examinations. Following this session, Georgia Tech personnel responsible for conducting the unscheduled necropsies will be fully trained.

Dr. Kovatch will also examine all test animals to determine their health status and will monitor the routine aspects of the daily animal care procedures. Ms. Sexsmith, as part of her quality assurance responsibilities, will assure that all SOPs have been appropriately modified and are in place and functional. She will also assure that appropriate study files and record systems have been established and are operational. Ms. Sexsmith will monitor the necropsy of sentinel animals, the animal identification procedures, routine animal care/husbandry practices, and the procedures associated with the first day of actual RFR exposure.

3. Radiation Logistics

The quarantine schedule will be arranged such that the RFR exposure begins (Time Point 0) on a Monday. At this Time Point, the transmitter will be energized at 12:00 noon and adjusted such that the exposure field results in a dose of approximately 0.32 W/kg. This exposure will continue for 20 hours, with an electrical connection between the light timer and the transmitter used to de-energize the transmitter at 8:00 a.m. This exposure schedule will be maintained 7 days each week for 77 weeks. During the 8:00 a.m. to 12:00 noon period each day, routine facility cleaning, animal inspection, and transmitter maintenance will be accomplished in accordance with SOPs. Therefore, the facility will be swept daily; 100 mice will be removed from their exposure cages, weighed, and returned to clean cages daily (Mondays through Thursdays); all mice will be visually inspected and appropriate entries will be made in each animal's record daily; transmitter operation will be checked daily; soil trays under each cage will be changed every other day; all mice will be weighed weekly (while their cages are being washed) and weights will be entered into the computer; the watering system will be refilled weekly; etc.

At Time Point 6 months (6 months after exposure initiation), Dr. Kovatch and Ms. Sexsmith will return to Georgia Tech. Dr. Kovatch will supervise the third scheduled necropsy of sentinel animals during which time he will review the necropsy techniques used by Georgia Tech personnel. Samples will again be taken for serology, bacteriology, and parasitology examinations. He will also review in-life records and examine the tissue and necropsy records of unscheduled deaths recorded during the 6 months. For the unscheduled deaths that have occurred, Dr. Kovatch will return the formalin-fixed tissues to PAI for processing and evaluation. His microscopic findings will be forwarded to Georgia Tech and to the U.S. Air Force within 3 weeks of receipt of the wet tissues.

Ms. Sexsmith will conduct a critical phase inspection of the in-life procedures and review in-life records for compliance with Good Laboratory Practice requirements. She will also monitor the necropsy of sentinel animals during this visit. Her findings will be provided to both Dr. Kovatch and Georgia Tech with action items identified.

At Time Point 12 months, the activities of PAI and Georgia Tech personnel at Time Point 6 months will be repeated. Dr. Kovatch will again supervise the necropsy of 10 sentinel animals, and he will clinically observe all test animals. This observation will include palpation for and/or verification of neoplasms. Dr. Kovatch will also observe in-life procedures conducted during his visit and examine the necropsy forms of animals that either died spontaneously or were moribund and euthanatized. In some cases, he may elect to

review the formalin-fixed wet tissues taken from selected unscheduled deaths. Based on the review of these tissues, he may elect to have additional formal training in necropsy procedures.

Ms. Sexsmith will conduct a quality assurance audit that includes monitoring necropsy procedures, in-life techniques, and reviewing all study records and files.

We estimated that approximately 40 animals will have died at this 12-month juncture. Tissues from these animals will be returned to the PAI laboratory for processing and evaluation. Dr. Kovatch will report and discuss results of the microscopic examination of these animals and the sentinel animals within 4 weeks of receipt of the wet specimens.

The RFR exposure will be terminated at Time Point 18 months. At this time, we estimated that 300 mice (approximately equal numbers of exposed and sham-exposed) plus 7-to-8 sentinel mice will remain. The scheduled necropsy of these animals will require approximately 5 days and will be accomplished over a Monday-through-Friday time period. Based on the 307-to-308 animal estimate for necropsy, PAI will provide 5 prosectors that will each necropsy an average of 12 mice daily. Two days before the necropsy, a van will leave the PAI-Hyattstown facility with all necessary supplies (specimen containers, formalin, instruments, etc.) to conduct the large-scale rodent necropsy.

The scheduled necropsy will be conducted by a PAI team comprised of Dr. Kovatch, 5 prosectors, and 1 necropsy technical assistant. The technical assistant will be responsible for animal euthanasia, initial verification of animal identification, and general assistance to the prosectors. The proposed method of euthanasia will be cervical dislocation. Ms. Sexsmith will monitor the scheduled necropsy as a critical phase in the study and will also review in-life records and study files. Hooded areas with exhaust to outside air will be provided at the work stations used by the 5 prosectors and Dr. Kovatch. This protection will prevent undesirable exposure of personnel to chemical fumes generated by the necropsy procedures.

Following completion of the scheduled necropsy, all specimens (from scheduled euthanasia sentinel animals, and unscheduled deaths) will be inventoried, packed into the van for safe transport, and returned to the PAI-Hyattstown laboratory for processing. A computerized in-processing inventory will be conducted of all materials (specimens and data) and they will be placed in the Working Archive in preparation for processing. We proposed that all aspects of histologic preparation will be completed within a 4-week period. Dr. Kovatch will evaluate all protocol-specified tissues and gross lesions. Microscopic findings will be entered into the PAI LABCAT automated pathology data system and a report formulated.

The PAI Quality Assurance Division in Hyattstown will monitor all critical phases of tissue preparation, evaluation, and report formulation. A Quality Assurance Certification Statement will accompany the final Pathology Report.

J. STUDY DOCUMENTATION

Documentation resulting from this study will be a three-volume final report as follows:

Volume I: Engineering Report (similar to reference [9]),

Volume II: In-Life Report, and

Volume III: Pathology Report.

The Engineering Report will document all aspects of the study that were concerned with the design and operation of the Radiation Facility. This will include the exposure waveguides, cages, watering system, exposure field, dosimetry, daily/weekly maintenance schedules, engineering-related SOPs, etc. The introduction to this report will present the study purpose and overview. This introduction will be followed by sections that describe in detail the various engineering considerations important to the results of the study. The depth of detail will be sufficient to permit the study's engineering aspects to be repeated, if desired, at some future time.

The In-Life (Toxicology) Report will document the care provided the animals, body weight information, and in-life clinical observations.

The Pathology Report will be prepared upon completion of the histopathologic evaluations and data entry, and will present pertinent results, interpretations and evaluations. This report will consist of a narrative section that includes an introduction which defines the purpose and overview of the pathology portion of the study. This introduction will be followed by a definitions section in which the morphologic terminology used to describe and diagnose exposure-associated tissue changes are presented.

The results portion of the narrative will expand, clarify, and explain the study findings. In particular, any observed RFR-induced lesions will be identified and separated from "background" lesions. In preparing these results, the pathologist will consider other study data (clinical signs, food and water consumption, body weight, clinical pathology results, and mortality) in relation to histopathologic and gross observation findings. Whenever possible, the pathogenesis of any observed RFR-induced lesions will be elucidated.

Photomicrographs will be taken of any RFR-induced lesions, and will be included in the report along with a descriptive narrative for each photograph. These photographs will be provided using the 2 x 2 photographic slide format.

In a separate notebook, an index and catalog of all photographs taken during the study will be presented including those microscopic findings that demonstrate typical RFR-associated tissue changes. All photographs will be numbered to correspond to the index that contains the specific diagnosis. The diagnosis and pertinent identification data will also be written on the slide mount.

The final portion of the Engineering, In-Life, and Pathology Reports will be a separate Quality Assurance section. This section will include a Certification Statement prepared by either the director or a member of the Quality Assurance Unit. Also included in this section will be a narrative that addresses the critical phase inspection conducted and certification that the study was conducted in compliance with EPA Good Laboratory Practice standards.

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